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RESPONSE PREDICTION IN CLEAR CELL RENAL CELL CARCINOMA TREATED WITH ANTI-VASCULAR ENDOTHELIAL GROWTH FACTOR-TARGETED THERAPY

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The initiation of this PhD project goes back without any doubt to my time in Paris, from April 2009 to July 2010, which was at the end of my fellowship in Medical Oncology. In April 2009, Professor Stéphane Oudard gave me the opportunity, first as an intern, and later in October 2009 after obtaining my degree in Medical Oncology, as a staff member, to work at the Medical Oncology Department of the Hôpital Européen Georges Pompidou, associated with the University Paris-5 René Descartes. Professor Stéphane Oudard sparked my interest in renal cell carcinoma and provided me access to the tumour bank that he had been collecting since 2007 at the INSERM research unit U674 "Génomique fonctionelle des tumeurs solides", headed by Professor Jessica Zucman-Rossi. Professor Olivier Rixe, at that time a Medical Oncologist at the Paris Hospital Pitié-La Salpétrière, obtained the grant of the Fondation Martine Midy in Bobigny that allowed me to start this research project. Professor Stéphane Oudard also gave me access to the clinical database of metastatic renal cell carcinoma patients treated at his institution. This first database became the backbone of a database of over 500 metastatic renal cell carcinoma patients treated with anti-angiogenic therapy.

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Benoit Beuselinck, Leuven, May 2014

ABBREVIATIONS

ANG: angiopoietin
ASL: arterial spin labeling
BAP1: breast cancer associated protein-1
CAIX: carbonic anhydrase IX
ccRCC: clear cell renal cell carcinoma
CRP: C-reactive protein
CXCR4: chemokine-CXC-motif-receptor-4
ECM: extracellular matrix
ECOG PS: Eastern Cooperative Oncology Group Performance Status
EGFR: epithelial growth factor receptor
EMT: epithelial-to-mesenchymal transition
FGF: fibroblast growth factor
FGFR: fibroblast growth factor receptor
FIH: factor inhibiting HIF
GIST: gastro-intestinal stromal tumours
GLU1: glucose-transporter protein-1
HGF: hepatocyte growth factor
HIF: hypoxia induced transcription factor
IFN-alpha: Interferon alpha
IHC: immunohistochemistry
ILGF: insulin-like-growth factor
IL2: interleukin-2
IL6: interleukin-6
IL8: interleukin-8
IMDC: International Metastatic Renal Cell Carcinoma Database Consortium
LDH: lactate dehydrogenase
LOH: loss of heterozygosity
m-ccRCC: metastatic clear cell renal cell carcinoma
MET: mesenchymal-to-epithelial transition
MMP: matrix metalloproteinase
mOS: median overall survival
mPFS: median progression-free survival
mRCC: metastatic renal cell carcinoma
MRI: magnetic resonance imaging
MSKCC: Memorial Sloan Kettering Cancer Center
mTOR: mammalian target of rapamycin
mTTP: median time-to-tumour-progression
MVD: micro-vessel density
MXI1: MAX interactor-1

OS: overall survival

PBRM1: polybromo-1

PDGF: platelet derived growth factor

PDGFR: platelet derived growth factor receptor

PFS: progression-free survival

PI3K: phosphatidylinositol 3-kinase

PLGF: placenta like growth factor

PTEN: phosphatase and tensin homolog

RCC: renal cell carcinoma

RECIST: Response Evaluation Criteria in Solid Tumours

RR: response rate

SDF1: stromal cell-derived factor-1

SETD2: SET domain containing-2

siRNA: small interfering RNA

TCEB1: transcription elongation factor B-1

TGF-alpha: transforming growth factor alpha

TGF-beta: transforming growth factor beta

TKI: tyrosine kinase inhibitor

TNM: Tumour Node Metastasis

TTP: time-to-tumour-progression

UMPP: ubiquitin-mediated proteolysis pathway

VEGF: vascular endothelial growth factor

VEGFR: vascular endothelial growth factor receptor

VHL: von Hippel-Lindau

WBMRI: whole-body magnetic resonance imaging

GENERAL INTRODUCTION: CURRENT STATE OF RESPONSE PREDICTION IN CLEAR CELL RENAL CELL CARCINOMA TREATED WITH ANTI-VEGF-TARGETED THERAPY

Until 2005, metastatic clear cell renal cell carcinoma (m-ccRCC) was a disease difficult to treat: it was resistant to chemotherapy and immunotherapy seemed only to be helpful in a small subgroup of patients difficult to identify in advance. Since 2005, targeted therapy directed against the vascular endothelial growth factor (VEGF)-pro-angiogenic pathway has replaced immunotherapy. Targeting the VEGF-pathway was based on the discovery that clear cell renal cell carcinomas (ccRCCs) are highly hyper-vascularized tumours, probably due to the very frequent dysregulation of the von Hippel-Lindau (*VHL*) gene leading to amplification of hypoxia induced factor (HIF)-mediated gene transcription and up-regulation of VEGF-dependent angiogenesis (1).

VEGF-directed monoclonal antibodies such as bevacizumab and anti-VEGF-receptor (VEGFR)-tyrosine kinase inhibitors (TKIs) such as sunitinib, pazopanib, and sorafenib, have improved the outcome of m-ccRCC patients, significantly prolonging the median progression-free survival (PFS) compared to immunotherapy or placebo (2-4). Some 40% of patients will achieve a reduction of the size of their metastases or primary tumours. In an additional 40-45% of patients, there will be disease stabilization under the therapy. Moreover, long-lasting or complete responses are obtained in a limited number of patients. Other compounds such as temsirolimus and everolimus, which are antagonists of the mammalian target of rapamycin (mTOR), another key protein in the regulation of intracellular signal transmission, have shown activity in the treatment of metastatic RCC (mRCC) (5, 6). Nevertheless, despite the convincing results of anti-VEGF-targeted therapy, some 15-20% of the patients are primarily resistant to these compounds and most of the patients who initially respond to the therapy will eventually develop secondary resistance and relapse after more or less one year of treatment (7). If the tumour is resistant from the start of the therapy, this is called “primary resistance”. If the tumour develops resistance after some time on anti-VEGF-targeted therapy, this is called “secondary resistance”.

In several other tumour types molecular markers predictive for response on targeted therapies have been discovered: *BRAF*-mutations for vemurafenib in melanoma, *c-KIT*-mutations for imatinib in gastro-intestinal stromal tumours (GIST), *KRAS*-mutations for cetuximab and panitumumab in colorectal carcinoma, *EGFR*-mutations for gefitinib and erlotinib in non-small cell lung carcinoma, the *abl-bcl* fusion gene for imatinib and dasatinib in chronic myeloid leukaemia, *ALK*-mutations for crizotinib in non-small cell lung carcinoma, and *HER2/neu*-amplification for trastuzumab or lapatinib in breast cancer. Unfortunately, there are no validated markers predicting response in m-ccRCC patients treated with anti-VEGF- or mTOR-targeted therapy.

Several clinical markers associated with PFS and overall survival (OS) were described and validated. They have been combined in scores such as the Memorial Sloan Kettering Cancer Center (MSKCC) (8) and International Metastatic Renal Cell Carcinoma Database Consortium (IMDC) score (9), described in paragraph 5 of the general introduction, but they have a prognostic rather than a predictive value. The clinical and biochemical parameters described until today do not allow us to predict resistance in individual patients nor to preclude patients from anti-VEGF-targeted therapy.

Therefore, the aim of this project was to investigate a variety of clinical, biochemical, pathological, and molecular or genetic markers of potential prognostic and/or predictive value in the context of anti-VEGFR-TKIs in m-ccRCC patients. Reliable response prediction could help us to better define the prognosis for a given patient and to improve our understanding of the mechanisms of primary and secondary resistance. This will eventually allow us

to improve the outcome of m-ccRCC patients by a more rational use of targeted therapies, possibly avoiding unnecessary adverse events in resistant patients and potentially saving costs. Some mechanisms of resistance may be relevant to the whole class of anti-VEGF-targeted therapies, others will likely only apply to individual compounds. In that case, a better understanding of the mechanisms of resistance will also allow us to define the best therapeutic options and help us to optimize the sequence of the available treatments.

1. EPIDEMIOLOGY, CLINICAL COURSE, DIAGNOSTIC AND THERAPEUTIC APPROACH IN RENAL CELL CARCINOMA

Renal Cell Carcinoma (RCC) accounts for about 2-3% of all adult malignancies, representing the seventh most common cancer in men and the ninth most common cancer in women (10). There were 46.151 new cases in the European Union in 2008 (11). In Belgium, in 2010, 1.532 patients were diagnosed with kidney cancer. RCC is approximately 50% more common in men compared with women and occurs predominantly in the sixth to eighth decade of life with a median age at diagnosis around 64 years. In developed countries, RCC is the tenth most common cancer-related cause of death.

RCCs, which originate within the renal cortex, constitute 80 to 85% of primary renal neoplasms and present several distinct subtypes, including ccRCCs (75-85%), papillary or chromophilic RCCs (10-15%), chromophobe RCCs, oncocytomas and collecting duct carcinomas (Bellini duct) (12). Fuhrman et al. described the most commonly used gradation of ccRCCs (grade 1 to 4) (13).

Many patients are asymptomatic until the disease is in a more advanced stage. The most common presenting symptoms are haematuria, abdominal mass, pain, and weight loss. Haematuria (in up to 40% of patients) is observed with tumour invasion of the collecting system. Fever (in 20% of patients) is usually intermittent and frequently accompanied by night sweats, anorexia, weight loss, and fatigue. Inferior vena cava involvement can produce lower extremity oedema, ascites, hepatic dysfunction and pulmonary emboli. Among patients with disseminated disease, signs or symptoms depend on the site and extent of the metastases. The most common sites are lung (70%), regional and thoracic lymph nodes (60%), bone (35%), liver (25%), brain (7%), the ipsilateral adrenal gland and the contralateral kidney. Paraneoplastic symptoms are not uncommon: anaemia, erythrocytosis (present in 1 to 5% of patients due to constitutive production of erythropoietin), thrombocytosis, hepatic dysfunction and hypercalcemia.

Around three out of five RCC-patients present with localized disease (confined to the kidney), one out of five with regional disease (spread to regional lymph nodes) and one out of five with metastatic disease. A rise in the incidence of localized disease and a reduction in the incidence of regional and metastatic spread as well as a steady decrease in the size of tumours at presentation, were seen during the last years, likely due to the greater number of incidental tumours detected on abdominal imaging performed for other purposes.

Although with state-of-the-art imaging techniques the histological diagnosis of solid renal masses can be predicted in many cases, a definite histological diagnosis can only be achieved by analysis of a biopsy or a specimen. For patients with isolated, solid renal masses, resection with either a partial or complete nephrectomy is preferred to biopsy, because it provides both the diagnosis and serves as definitive treatment. When metastatic disease is suspected at initial presentation, pathologic confirmation of at least one disease site is required prior to starting therapy. Biopsy of a metastatic site is often easier and more informative than biopsy of the primary

tumour. The Tumour Node Metastasis (TNM) staging system, revised in 2010 (14), defines the anatomic extent of disease and stage (Table 1 and 2).

PRIMARY TUMOUR (T)	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
T1	Tumour 7 cm or less in greatest dimension, limited to the kidney T1a: Tumour 4 cm or less in greatest dimension, limited to the kidney T1b: Tumour more than 4 cm but not more than 7 cm in greatest dimension, and limited to the kidney
T2	Tumour more than 7 cm in greatest dimension, limited to the kidney T2a: Tumour more than 7 cm but less than or equal to 10 cm in greatest dimension, limited to the kidney T2b: Tumour more than 10 cm, limited to the kidney
T3	Tumour extends into major veins or perinephric tissues but not into the ipsilateral adrenal gland and not beyond Gerota's fascia T3a: Tumour grossly extends into the renal vein or its segmental (muscle containing) branches, or tumour invades perirenal and/or renal sinus fat but not beyond Gerota's fascia T3b: Tumour grossly extends into the vena cava below the diaphragm T3c: Tumour grossly extends into the vena cava above the diaphragm or invades the wall of the vena cava
T4	Tumour invades beyond Gerota's fascia (including contiguous extension into the ipsilateral adrenal gland)
REGIONAL LYMPH NODES (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in regional lymph node(s)
DISTANT METASTASIS (M)	
M0	No distant metastasis
MI	Distant metastasis

Table 1: TNM staging system for kidney cancer

STAGE	TUMOUR	LYMPH NODES	METASTASES
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T1 or T2	N1	M0
	T3	N0 or N1	M0
Stage IV	T4	Any N	M0
	Any T	Any N	M1

Table 2: Anatomic staging/prognostic groups

For localized resectable RCC, surgery can be curative in the majority of patients. Forty percent will relapse with either local and/or metastatic disease (15)(16). When distant metastases occur, some patients with a limited amount of lesions could be cured with metastasectomy or local radiation therapy, but the majority will require systemic therapy.

In patients with metastatic disease at initial diagnosis or locally advanced non-resectable disease, cytoreductive nephrectomy remains standard of care. With a response rate (RR) of only 5 to 10%, classical chemotherapy with cytotoxic drugs does not have an established role in the management of mRCC patients. Only subgroups of

patients can occasionally respond to chemotherapy and the quality of responses is usually poor. The introduction of immunotherapy with either interleukin-2 (IL2) or Interferon (IFN)-alpha slightly improved the outcome of mRCC patients through immune-modulatory effects. Immunotherapy with high-dose bolus IL2 results in tumour regressions in a minority (10%) of patients. Although this treatment is associated with severe toxicity, responses may persist for many years, even in the absence of additional therapy, and the majority of complete responders remain free of relapse for prolonged periods of time. Monotherapy with IFN-alpha in mRCC leads to RR in the range of 15%, most responses are partial and rarely persist beyond one year. Treatment with IFN-alpha is associated with an average median improvement in survival of four months (17).

Over the last 10 years, molecularly targeted therapy directed against the VEGF-pathway has appeared on the scene with several new compounds such as the anti-VEGFR-TKIs sunitinib, sorafenib, pazopanib and axitinib and the monoclonal anti-VEGF-antibody bevacizumab. Moreover, two mTOR inhibitors, everolimus and temsirolimus, have also become available. These compounds have largely replaced the use of immunotherapy for patients with advanced RCC since 2005 because of demonstrated improved efficacy. Sunitinib and pazopanib are the most commonly used oral drugs for first-line anti-VEGF-targeted therapy for mRCC. Table 3 shows the results of pivotal phase III trials that have established the efficacy of these compounds in kidney cancer.

The efficacy of sunitinib in m-ccRCC was shown in a phase III trial of 750 patients with largely good or intermediate MSKCC prognosis who had not received any prior systemic therapy. Patients were randomly assigned to sunitinib or IFN-alpha. The RR was significantly increased with sunitinib (47 *versus* 12%) and median PFS (mPFS) was significantly prolonged (11 *versus* 5 months, $p<0.001$). OS was 26.4 months with sunitinib *versus* 21.8 months with IFN-alpha ($p=0.051$) (3, 18). In a phase III trial, 435 patients, with mainly clear cell histotype, good or intermediate MSKCC risk, who were previously untreated or had received only cytokine therapy, were randomly assigned to pazopanib or placebo. There was a significant increase in mPFS with pazopanib compared with placebo (9.2 *versus* 4.2 months, HR 0.46). Median OS (mOS) was non-significantly different (22.9 *versus* 20.5 months, HR 0.91), but these results are confounded by the high rate of cross-over and/or use of other treatments after initial evidence of progressive disease in patients included in the placebo arm of the trial (4). Two phase III trials compared sunitinib to pazopanib in first-line therapy. The first trial showed non-inferiority of pazopanib in terms of PFS (19). The second, much smaller trial demonstrated patient preference for pazopanib in a randomized, double blind setting.

In second-line setting, axitinib has been studied in a randomized phase III trial that included 723 m-ccRCC patients who had prior treatment with a cytokine (35%) or another anti-VEGF or mTOR-targeted treatment (54% with sunitinib, some with bevacizumab or temsirolimus). Compared to sorafenib, axitinib resulted in a significant improvement in mPFS (7 *versus* 5 months). The benefit in PFS was higher in patients previously treated with cytokines (12 *versus* 7 months) than in patients previously treated with sunitinib (5 *versus* 3 months). There was also a significant increase in RR (19 *versus* 9%) (20).

TRIAL	INCLUDED PATIENTS	PROGNOSTIC GROUPS (MSKCC)	PFS (MONTHS)	OS (MONTHS)	RR EXPERIMENTAL ARM
EFFICACY OF TARGETED THERAPIES IN PATIENTS PRE-TREATED WITH CYTOKINES					
Sorafenib *versus* placebo TARGET	Clear cell	Good 52% Intermediate 48% Poor 0%	5.5 *versus* 2.8 p<0.01	17.8 *versus* 15.2 NS	PR 10% SD 74% PD 16%
Pazopanib *versus* placebo	Clear cell (or predominantly)	Good 39% Intermediate 55% Poor 3%	7.4 *versus* 4.2 p<0.001	Cross-over	PR 33% SD 42% PD 16%
Axitinib *versus* sorafenib AXIS	Clear cell	Not available	12.1 *versus* 6.5 p<0.0001	Cross-over	NR
EFFICACY OF FIRST-LINE TARGETED THERAPIES IN TREATMENT NAIVE PATIENTS					
Sunitinib *versus* interferon	Clear cell	Good 38% Intermediate 56% Poor 6%	11 *versus* 5.1 p<0.001	26.4 *versus* 21.8 p=0.051	CR 3% PR 44% SD 40% PD 7%
Temsirolimus *versus* Interferon	(Non) clear cell	Intermediate 31% Poor 69%	5.5 *versus* 3.1 p<0.001	10.9 *versus* 7.3 p=0.008	CR 0% PR 9% SD 46%
Bevacizumab+Interferon *versus* Interferon+placebo AVOREN	Clear cell or clear cell component	Good 29% Intermediate 56% Poor 8%	10.2 *versus* 5.5 p=0.0001	23.3 *versus* 21.3 NS	CR 1% PR 30% SD 46% PD 20%
Bevacizumab+Interferon *versus* Interferon+placebo CALGB	Clear cell	Good 26% Intermediate 64% Poor 10%	8.5 *versus* 5.2 p<0.0001	18.3 *versus* 17.4 NS	PR 26%
Pazopanib *versus* placebo	Clear cell or clear cell component	Good 39% Intermediate 55% Poor 3%	11.1 *versus* 2.8 p<0.001	Cross-over	PR 32%
EFFICACY OF SECOND-LINE TARGETED THERAPIES IN PATIENTS PROGRESSING ON FIRST-LINE TKIs					
Everolimus *versus* placebo RECORD-1	After TKI failure Clear cell component	Good 29% Intermediate 56% Poor 15%	4.0 *versus* 1.9 p<0.0001	Cross-over	PR 1% SD 63% PD 36%
Axitinib *versus* sorafenib AXIS	Second-line after sunitinib Clear cell	Not available	4.8 *versus* 3.4 p=0.0107	Cross-over	Axitinib: PR 11.3% Sorafenib: PR 7.7%

Table 3: Overview of phase III trials with targeted therapy for mRCC. NS: not significant. NR: not reported.

The efficacy of sorafenib in advanced ccRCC was demonstrated in a phase III trial, in which 903 patients who had failed prior cytokine therapy were randomly assigned to sorafenib or placebo. mPFS was significantly longer in those receiving sorafenib compared with placebo (5.5 *versus* 2.8 months). mOS was not significantly prolonged (17.8 *versus* 15.2 months), but patients were allowed to cross-over and receive sorafenib, potentially obscuring survival differences (2, 21).

Bevacizumab is a monoclonal antibody that binds circulating VEGF and prevents its interaction with the VEGFR. Two phase III trials randomized 649 and 732 previously untreated m-ccRCC patients in two treatment arms: bevacizumab plus IFN-alpha compared with IFN-alpha alone. In the first trial (22-25), the combination resulted in an increase in mPFS (10.2 *versus* 5.5 months), a significantly higher RR (31 *versus* 13%) and a trend toward

improved mOS (23.3 *versus* 21.3 months). The second trial reported similar results (26). In both studies, the survival analysis was biased by the fact that more than one-half of patients on both arms received second-line therapy, including VEGF-targeted therapy in those originally treated with IFN-alpha alone.

The mTOR-pathway is downstream of the phosphoinositide-3-kinase and AKT-pathway that is regulated by the *PTEN* tumour suppressor gene. Inhibition of the mTOR-pathway by temsirolimus or everolimus has the potential to inhibit tumour progression at multiple levels. In randomized trials, the mTOR-inhibitors temsirolimus and everolimus have shown clinical activity in mRCC patients. Temsirolimus has shown efficacy in first-line MSKCC poor risk mRCC (5, 27) and everolimus in second-line therapy after progression on anti-VEGF-targeted therapy (6, 28)

As a consequence, within 8 years, 7 compounds were approved for the treatment of mRCC. This raises questions regarding the optimal use of these agents, including which agent to use in a particular patient, what is the optimal sequence of available agents and if concomitant use of these therapies can lead to improved outcomes. Several trials investigated the combined use of agents hitting the VEGF- and the mTOR-pathway. These trials consequently led to increased toxicity, but not to increased efficacy. Concerning sequential use of these compounds, axitinib and everolimus are the two molecules with phase III data proving efficacy in patients who progressed on a first-line therapy with TKIs. Temsirolimus, sunitinib and sorafenib can also be given in second-line. Nevertheless, the global efficacy of second-line therapy is rather disappointing, with shorter PFS, fewer PRs and no long lasting disease control in the vast majority of patients. Retrospective studies as well as a randomized phase III trial comparing sorafenib and temsirolimus after sunitinib did not show any advantage of switching to mTOR-inhibition compared to continuing a second anti-VEGF-targeted therapy (29).

Surgery and radiation therapy may be used in carefully selected mRCC patients. Despite the characterization of RCC as a relatively radio-resistant tumour, conventional and stereotactic radiation therapy can be used to treat symptomatic bone lesions or brain metastases. The resection of a limited number of metastases can be considered in combination with nephrectomy or at subsequent relapse in carefully selected patients for both pain relief and tumour control. The reported outcomes after resection have been variable, with five-year survival in small series ranging from 13 to 50%. Selection bias has an important impact on such reported results.

In patients qualifying for systemic therapy, immediate start of treatment or a watchful waiting policy can be considered. Some RCCs can have a very indolent course and spontaneous regression of metastasis is observed in individual patients. (30).

2. PATHOPHYSIOLOGY OF CLEAR CELL RENAL CELL CARCINOMA

ccRCCs arise from cells of the proximal tubule, either sporadically, or associated with three distinct cancer syndromes: the Von Hippel-Lindau (*VHL*) disease, hereditary paraganglioma and pheochromocytoma and tuberous sclerosis complex. The most common genomic alterations in ccRCC are loss of chromosome 3p and mutations in the *VHL* gene located on 3p25-26 or hyper-methylation of its promoter. The initial insights into the molecular pathogenesis of ccRCC came from studies in VHL-disease, where VHL-associated RCCs showed loss of heterozygosity (LOH) at the *VHL*-locus.

VHL-disease is an inherited, autosomal dominant syndrome associated with a variety of benign and malignant tumours: haemangioblastomas of the cerebellum and spine, retinal angiomas, multiple renal cysts and ccRCCs

(in 66% of patients), pheochromocytomas, endolymphatic sac tumours of the middle ear, serous cystadenomas, neuroendocrine tumours of the pancreas and papillary cystadenomas of the epididymis and broad ligament. A *VHL*-gene abnormality is present in about 1 in 36.000 individuals. Affected patients have a germ-line mutation that inactivates one copy of the *VHL*-gene in all cells. For disease to occur there must be loss of expression of the second, normal allele (31).

In sporadic ccRCC, *VHL*-gene inactivation occurs in approximately 60-70% of the cases, mostly through mutations (55-60%), but in 10-15% of cases through promoter hyper-methylation (32-35). In the majority of tumours that show somatic *VHL*-mutations, the other allele is deleted by LOH. Other reports using high throughput methodologies allowed to improve identification of *VHL*-alterations: up to 91% of patients with ccRCC patients harbour a *VHL*-gene alteration through genetic or epigenetic mechanisms (36). Moreover, introduction of a 3p chromosome fragment in ccRCC cell-lines suppresses tumorigenicity (37).

VHL-gene inactivation seems to be an early event in ccRCC carcinogenesis. This is suggested by the frequency of this event and the fact that there is no link between mutational status *versus* hyper-methylation *versus* none of both and the clinical characteristics of the tumours.

The VHL-protein controls the metabolism of HIF. Other functions of the *VHL*-protein are fibronectin assembly, microtubule stability, atypical protein kinase C activity and p53 regulation in an HIF-independent way (38).

2.1. VHL inactivation leading to hyper-vascular tumours through hypoxia-inducible factor (HIF) induction

HIF as a transcription factor and a key regulator of hypoxia-inducible genes regulates more than 100 genes, controlling angiogenesis, cell cycle induction, apoptosis, glucose metabolism, erythropoiesis, macrophage function, extracellular matrix (ECM) remodelling, tumour growth factors and receptors and cell migration (39).

HIF is a heterodimer consisting in an alpha subunit (HIF1-alpha or HIF2-alpha), sensitive to oxygen levels, bound by the VHL-protein, and a beta subunit (HIF1-beta or aryl hydrocarbon receptor nuclear translocator), not influenced by the oxygen tension and not bound by the VHL-protein complex. Under normoxic conditions, HIF1-alpha is hydroxylated which creates a binding site for the VHL-protein leading to an E3-ubiquitin ligase-mediated degradation in the proteasome. In the presence of oxygen, the half-life of HIF1-alpha is less than 5 minutes. Under hypoxic conditions, there is no HIF hydroxylation and hence no ubiquitination and no degradation. The combination of HIF1-beta and HIF1-alpha or HIF2-alpha becomes a transcription factor that accumulates in the nucleus and binds on hypoxia responsive elements inducing the transcription of numerous genes as a physiologic response to hypoxia. Nevertheless, once this physiologic response has restored normoxia in the tissue, HIF will be degraded again by the VHL-protein. In case of loss of VHL-protein function, HIF cannot be degraded anymore and the downstream HIF dependent genes will remain up-regulated even in restored normoxic conditions.

HIF is clearly up-regulated in tumours with VHL-loss. In one study, increased expression of HIF was recorded in 75% of ccRCC and only 38% of non–ccRCC cases (40). In another study, the level of HIF appeared to correlate with the presence of *VHL*-mutation and none of the HIF-negative ccRCC patients showed a mutation of the *VHL*-gene. ccRCC had a significantly higher HIF-expression compared to papillary or chromophobe RCC variants (41).

HIF induces cellular multiplication through three pathways: (A) activation of c-myc, which induces the cell cycle by the activation of cyclin-D2 and E2F1 and the repression of p21 and p27, (B) c-myc mediated mismatch repair, which is necessary to compensate for replication stress in high turnover cells, (C) transcription of growth factors and receptors like epithelial growth factor receptor (EGFR) (expressed on RCC cells) and its ligand transforming

growth factor (TGF)-alpha, chemokine-CXC-motif-receptor-4 (CXCR4) (expressed on RCC cells) and its ligand stromal derived factor-1 (SDF1), insulin-like growth factor (ILGF) and its binding protein and TGF-beta. The over-expressed and activated CXCR4 and EGFR will activate the RAS/RAF/ERK/MAPK1 pathway, leading to cell cycle induction, and the RAS/PI3K/PDK/AKT/mTOR-pathway, leading to HIF-activation.

HIF promotes neo-angiogenesis by activating several genes: *VEGF* and its receptor *VEGFR1*, *-2* and *-3*, *PDGF* and its receptors *PDGFR-alpha* and *-beta*, *fibroblast growth factor (FGF)-2* and *FGF5*, *angiopoietin-2 (ANG2)* and its receptor *Tie2*, *plasminogen activator inhibitor-1*, *SDF1* and *hepatocyte growth factor (HGF)*. HIF also induces several genes linked to glucose transportation and metabolism, necessary for energy delivery to multiplying cells, as well as invasiveness genes, like matrix metalloproteinases (MMPs). MMPs are necessary for normal angiogenesis, because they have to cut the basal membrane allowing the penetration of new vessels. This rupture of the basal membrane will also permit cell migration into the blood circulation, thus promoting metastasis in case of cancer.

In case of VHL-protein dysfunction, restored normoxia will not lead to HIF-degradation and HIF will pursue its activity that will even become auto-reinforcing through an autocrine loop. Moreover, due to insufficient neo-angiogenesis and uncontrolled cellular growth, there will be enhanced interstitial pressure and vascular compression, which will again lead to hypoxia and HIF-activation. These auto-reinforcing loops are probably at the origin of uncontrolled cellular multiplication and thus tumorigenesis. Adding neo-angiogenesis, enhanced glucose transportation and rupture of the basal membrane by MMPs, all the conditions for tumoral growth and metastasis are fulfilled.

Of note, HIF can also be activated in cancer by micro-environmental hypoxia in hypoxic tumour zones, without *VHL* silencing (42).

2.2. HIF1-alpha and HIF2-alpha subtypes

HIF1-alpha and HIF2-alpha have overlapping effects on angiogenesis and ECM remodelling, but distinct effects on cell metabolism and proliferation. HIF1-alpha activates the transcription of the *MXI1*-gene, which encodes a repressor of c-myc transcriptional activity, and promotes a *MXI-1*-independent proteasome dependent degradation of c-myc (43, 44). Cyclin D is down-regulated by HIF1-alpha. HIF1-alpha enhances transcription of BNIP3, a BCL2-related promoter of apoptosis. As a consequence, HIF1-alpha opposes cell cycle progression, induces cell arrest and opposes c-myc mediated mismatch repair. Glucose-transporter protein-1 (GLU1), a regulator of glucose metabolism, is down-regulated by HIF1-alpha (45).

HIF2-alpha induces the c-myc oncoprotein and c-myc mediated expression of cyclin-D2, cyclin-G2 and E2F1 and thus cellular proliferation. HIF2-alpha enhances DNA-mismatch repair and the expression of TGF-alpha, inducing an autocrine loop targeting the EGFR, MMP, GLU1, ANG2 and its receptor Tie2 and VEGF (42, 46). BNIP3 is down-regulated by HIF2-alpha (45).

This differential transcriptional activity of HIF1-alpha and HIF2-alpha attributes more aggressive genes to the influence of HIF2-alpha and confirms its important role in tumorigenesis. The HIF2-alpha subunit is primarily responsible for the growth of VHL-deficient human ccRCC xenografts. siRNA-mediated down-regulation of HIF2-alpha suppresses tumour formation by VHL-defective RCC cells (47). Finally, in a series of 160 tumours, not any tumour expressed HIF1-alpha without HIF2-alpha, strongly suggesting that HIF2-alpha is critical for the development of VHL-deficient ccRCC (48).

2.3. Neo-vascularisation in mRCC

When a tumour or a metastasis grows and cells in the centre of the tumour mass become hypoxic, the tumour initiates recruitment of its own blood supply, by shifting the balance between physiological angiogenesis inhibitors and stimulators towards the latter, a process called "angiogenic switch".

ccRCC are among the most hyper-vascular tumours in which pro-angiogenic mechanisms such as the VEGF- and/or other pro-angiogenic pathways are hyper-activated (Figure 1). On pathological analysis, ccRCC have significantly higher micro-vessel counts than non-ccRCC and these counts are correlated with the level of VEGF-expression (42). Enhanced VEGF-concentration occurs in RCC patients with *VHL*-gene alterations and advanced grade (49, 50).

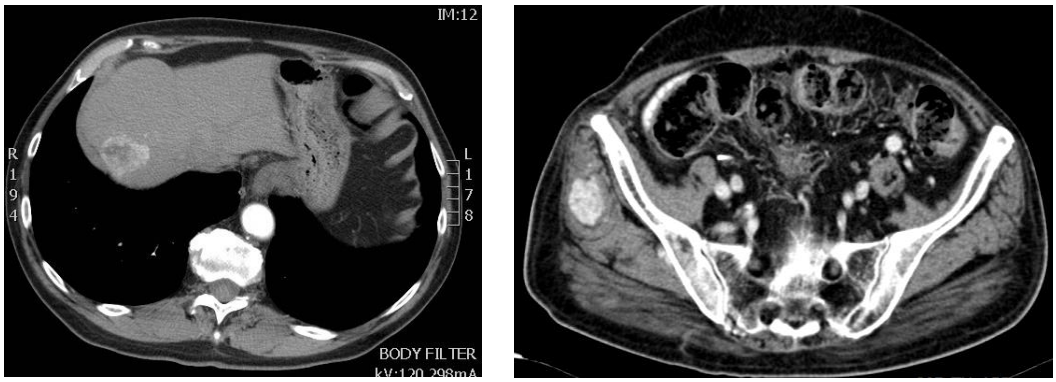

Figure 1. Contrast enhanced arterial phase CT of two patients with metastases from ccRCC. The liver metastasis at the left and soft tissue metastasis at the right display contrast enhancement due their high level of vascularization.

2.4. VEGF, the dominant growth factor controlling angiogenesis

Although there are multiple pro-angiogenic factors, the dominant growth factor controlling angiogenesis is VEGF (50, 51). VEGF-expression is highly regulated by hypoxia, providing a feedback mechanism to accommodate reduced tissue oxygenation via the promotion of new blood vessel formation. VEGF, produced by a number of different cell types, acts selectively on vascular endothelial cells, and is capable of stimulating angiogenesis *in vitro* and *in vivo*. It plays an active role in the induction, maintenance, and growth of vascular endothelial cells. VEGF also controls vascular permeability and is 50.000 times more potent in inducing vascular leakage than histamine. VEGF stimulates expression of tissue plasminogen activator, urokinase plasminogen activator, collagenases, and MMPs, involved in the degradation of the ECM, required for endothelial cell migration.

VEGF mediates angiogenic signals to the vascular endothelium via high affinity receptor tyrosine kinases, designated VEGFR1, -2, and -3. These receptors are expressed almost exclusively on endothelial cells. Neuropilin-1 has been proposed to function as a VEGFR2-co-receptor capable of enhancing the biological effects of VEGF on endothelial cells. Tip cells, which are the cell at the leading edge of an angiogenic sprout, are primarily responsible for sensing the chemo-attractive VEGF-gradients secreted by hypoxic tissue. The tip cells express high levels of VEGFR3 (50).

Neo-angiogenesis occurs in an activation and a maturation phase. The activation phase implies the destruction of the basal membrane around the existing vessel by MMPs, the recruitment, migration and proliferation of endothelial cells under influence of VEGF, the formation of a primitive tube of endothelial cells and the connection to existing vessels. Bone marrow-derived haematopoietic cells expressing markers such as CXCR4 and/or VEGFR1 become recruited, often together with endothelial progenitor cells, to tumours or ischaemic tissues in response to VEGF and placenta like growth factor (PlGF). The maturation phase leads to the stabilization of the developing vascularization by the reconstruction of a basal membrane and the recruitment of perivascular cells (pericytes) that will cover the new formed vessels. These mural cells differentiate from perivascular progenitor cells, which are mobilized from the bone marrow in response to PDGF-beta.

The VEGF-signalling pathway seems to be over-expressed early during ccRCC pathogenesis. In tumours, in contrast to other tissue, VEGF is not produced by the endothelial cells, but by tumour cells or stroma, consistent with a paracrine mode of action. When PDGFs are over-expressed, tumour vessels are covered by more mural cells and tumour growth is accelerated.

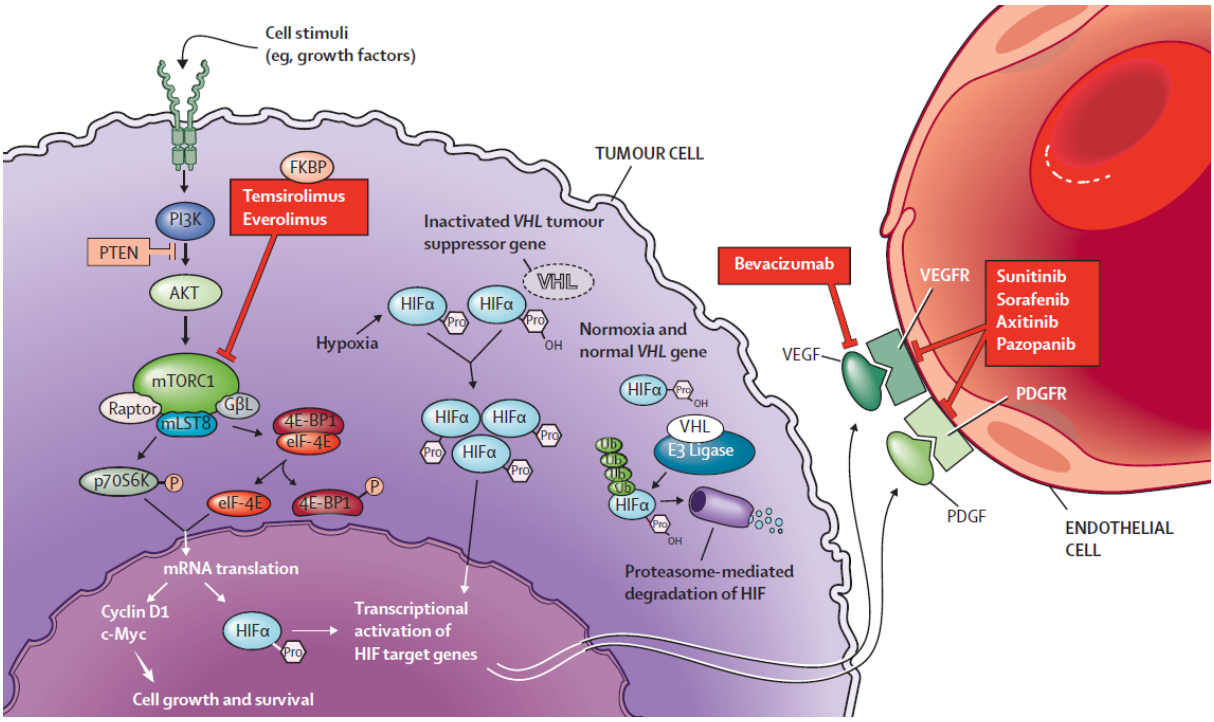

Figure 2. The involvement of VHL-dysfunction in the pathogenesis of ccRCCs and the resulting therapeutic targets. In conditions of normoxia and normal *VHL*-gene function, VHL-protein leads to the continuous degradation of HIF-alpha. In cellular hypoxia or with an inactivated *VHL*-gene, the VHL protein–HIF interaction is disrupted, leading to stabilization and accumulation of HIF transcription factors and the subsequent transcription of a large range of hypoxia-inducible genes, including *VEGF* and *PDGF*. These ligands bind to their receptors present on the surface of endothelial cells, leading to neo-angiogenesis. HIF also induces cell cycle through the induction of cyclin D1 and c-myc. The VEGF-activation can be blocked with the monoclonal anti-VEGF-antibody bevacizumab of with the anti-VEGFR-TKIs sunitinib, sorafenib, axitinib or pazopanib. Courtesy of Rini et al. and reproduced with permission from The Lancet Oncology. Copyright owned by Elsevier Ltd (7).

2.5. Other frequent mutations in ccRCC

Several other mutations have been described in ccRCC: *PBRM1* (in 41% of cases), *BAP1* (8-15%), *FAM123B* (13%), *CTNNB1* (16%), *WT1* (11%), *CDKN2A* (7-10%), *p53* (8%), *UTX* (*KDM6A*) (1.4-3%), *SETD2* (3-4%), *JARID1C* (*KDM5C*) (3-9%), *NF2* (5%) and *PTEN* (4%). Interestingly, several of these genes (*PBRM1*, *BAP1*, *SETD2*) are located on 3p and involved in chromatin organisation. Moreover, *VHL* and *BAP1* are members of the ubiquitin-mediated proteolysis pathway (UMPP), an important pathway for protein degradation through the proteasome. Alterations in genes encoding UMPP are associated with overexpression of HIF, even in the absence of *VHL*-mutation, thus they result in similar functional consequences as *VHL*-inactivation. In one study, UMPP was the most frequently altered pathway in ccRCC (51).

Polybromo-1 (PBRM1) is the second most mutated ccRCC gene, with truncating mutations in 41% of cases (52). *PBRM1* is likely a tumour-suppressor gene. *PBRM1* maps to chromosome 3p21 and encodes the BAF180 protein, the chromatin targeting subunit of the PBAF Switch/Sucrose Non Fermentable SWI/SNF chromatin remodelling complex (53). PBAF complex-mediated chromatin remodelling is implicated in replication, transcription, DNA repair and control of cell proliferation and differentiation. *PBRM1* activity regulates pathways associated with chromosomal instability and cellular proliferation. The SWI/SNF complex has been implicated in the normal cellular response to hypoxia, with impairment of the complex rendering cells resistant to hypoxia-induced cell cycle arrest (54).

The *BRCA1 associated protein-1 (BAP1)*, located at 3p, is a nuclear deubiquitinase part of the large UMPP (55). *BAP1*-mutant tumours are more likely to be aggressive and display adverse pathologic features, such as high Fuhrman grade, sarcomatoid and rhabdoid features of RCC, tumour necrosis, and mTOR Complex-1 activation. *BAP1*-mutant tumours are associated with changes in the expression of genes implicated in growth-factor signalling. Most genes that make up the *BAP1* signature are down-regulated in *BAP1*-mutant tumours. This raise the possibility that in the absence of *BAP1* transcription factors are ubiquitinated and targeted for proteosomal-mediated degradation. *BAP1* and *PBRM1*-mutations are mutually exclusive. mOS is significantly shorter for patients with BAP1-mutant tumours than for patients with *PBRM1*-mutant tumours and tumours exclusively mutated for *PBRM1* tend to be of lower grade (56).

SETD2 and *JARID1C*, in which the mutations are inactivating, encode enzymes involved in histone modification through demethylation, highlighting the important role of components of the chromatin modification machinery in RCC (57, 58). Sato et al. reported a poorer disease-free survival in patients with *SETD2*-mutated tumours (59).

VHL-mutations or loss of the VHL-function seem to be an early event in ccRCC pathogenesis, but not sufficient for the development of a tumour. Additional events are warranted. Mice bearing a *VHL*-mutation do not develop more often RCCs than the non-mutated counterparts. This is probably explained by the fact that in mice, *BAP1* and *PBRM1* are located on other chromosomes than *VHL* (60, 61). Both *BAP1* and *PBRM1* are two-hit tumour suppressor genes and they are located on chromosome 3p. The hypothesis is that, in many instances, the development of ccRCC is initiated by a focal mutation in *VHL*, followed by a 3p deletion. 3p loss may eliminate *VHL*-gene function and would leave cells with just one copy of *BAP1* and *PBRM1*. Mutation of the remaining *BAP1* or *PBRM1* allele may initiate tumorigenesis, resulting in tumours of different aggressiveness, depending on which gene is mutated. Thus, tumour aggressiveness may be established early on during the process of tumorigenesis (56).

2.6. Cytogenetic abnormalities, intratumoral heterogeneity, and ccRCC-subgroups based on expression profiles

In ccRCC, besides quasi omnipresent loss of 3p, there is a frequent gain of 5q (69% of cases), 7q (20-30%) and 8q (12%) and monosomy or partial loss of 14q (42%), 8p deletion (32%) and 9p loss (29%) (62).

There is evidence for significant intratumoral heterogeneity concerning frequently observed mutations in RCC. Differences in the mutational status can be found within one tumour or when comparing the original tumour with the metastases. Nevertheless, ubiquitous allelic-imbalance events were seen on chromosome 3p (encoding *VHL*, *PBRM1, BAP1* and *SETD2*), 5q, 6q and 10q (63).

Recently, important progress has been made in the identification of subgroups of ccRCCs through the analysis of gene-expression profiles and the additional analysis of epigenetic, cytogenetic and mutational characteristics of these tumours. In a series of 225 ccRCC, using traditional unsupervised gene expression analysis in a two-step analysis, with hybridization in a first group of samples, and validation by quantitative qRT-PCR on a second group of samples, Brannon et al. described two molecular sub-classifications of ccRCCs, called ccA and ccB (64). Both subgroups were confirmed in a meta-analysis of publicly available expression profiles of 480 ccRCCs, with the identification of an additional third group, called Cluster_3, and associated with a *VHL* wild-type profile (65). Brannon's ccA-tumours (51% of cases) showed overexpression of genes associated with hypoxia, angiogenesis, the beta-oxidation pathway, fatty acid metabolism, pyruvate metabolism, and organic acid metabolism. ccB-tumours (36% of cases) over-expressed a more aggressive panel of genes that regulate cell differentiation, epithelial-to-mesenchymal transition (EMT), the mitotic cell cycle, TGF-beta, wound healing and WNT. In ccB-tumours angiogenesis, beta-oxidation, pyruvate metabolism and organic and fatty acid metabolism were downregulated. ccB-tumours were more often Fuhrman grade 4. HIF signalling was found to be over-expressed in ccA relative to ccB tumours, although VHL-inactivation was observed by Brannon et al. in both clusters. Cluster_3 tumours (13% of cases) were characterized by over-expression of mitochondrial bioenergetics pathways and down-regulation of hypoxia and angiogenesis pathways. Histology was found to be reported ambiguously in this subgroup. More recently, four subgroups of ccRCCs and their associations with copy number abnormalities, epigenetic and mutational characteristics, were described by the TCGA-project (66) and by Sato et al. (59).

3. MECHANISM OF ACTION OF ANTI-ANGIOGENIC DRUGS

In order to understand the mechanisms of resistance to VEGF-targeted therapy, we should first understand the mechanisms of action of these therapies.

3.1. Inhibition of the VEGF- and PDGF-pathway

Anti-VEGFR-TKIs are thought to exert their major therapeutic effects in RCC by antagonising the VEGF-pathway, leading to reduced tumour angiogenesis (67). This concept is based largely on the significant up-regulation of VEGF in ccRCC as a result of VHL-inactivation, and the finding of anti-angiogenic effects in preclinical models (68-71). Moreover, anti-VEGF-targeted therapy is less efficient in non-ccRCCs (72-74), which are not associated with VHL-dysfunction.

Sunitinib, pazopanib, sorafenib and axitinib are orally available ATP-mimetic small molecule TKIs with tropism for the ATP-binding site of the VEGFR1, -2 and -3, although the specific affinity for these three receptors is variable. These compounds act as inhibitors of VEGFR signalling (75). The anti-VEGF-monoclonal antibody bevacizumab has a different mechanism of action: it captures circulating VEGF.

Additionally, several anti-VEGFR-TKIs block the PDGF-pathway: sunitinib and pazopanib through inhibition of both the PDGFR-alpha and -beta and sorafenib through the inhibition of PDGFR-beta. PDGFR-inhibition might enhance the anti-angiogenic effects of anti-VEGFR-TKIs by targeting pericytes, which are able to protect endothelial cells from apoptosis in the setting of VEGFR-blockade. Under anti-VEGFR-TKI-treatment, fewer pericytes are recruited, tumour vessels are dilated, endothelial cell apoptosis is increased, angiogenesis is suppressed, the interstitial fluid pressure is lowered and drug delivery improves (76).

3.2. Reduction of existing vessels, inhibition of new vessels and vessel normalization

The anti-tumour effects of VEGF-inhibition have been ascribed to the observed reductions in tumour micro-vessel density and tumour blood flow. Anti-VEGF-targeted therapies arrest endothelial cell proliferation, prevent vessel growth and induce regression of existing vessels by increasing endothelial cell death. They also suppress the mobilization of endothelial progenitor cells from the bone marrow and lower vessel permeability and thus tumour interstitial pressure.

VEGF-blockade as monotherapy has been clearly shown to have a direct and rapid anti-vascular effect in both animal and human tumours (77).

In RCC xenograft models, immunohistochemistry (IHC) analysis of tumours resected shortly after starting treatment with sorafenib showed a characteristic pruning of the microvasculature with necrosis. This devascularisation was visualised by arterial spin labeling (ASL) perfusion magnetic resonance imaging (MRI), showing a prompt and nearly complete cessation of blood flow shortly after initiation of sorafenib (Figure 3.1. and 3.2.) (71).

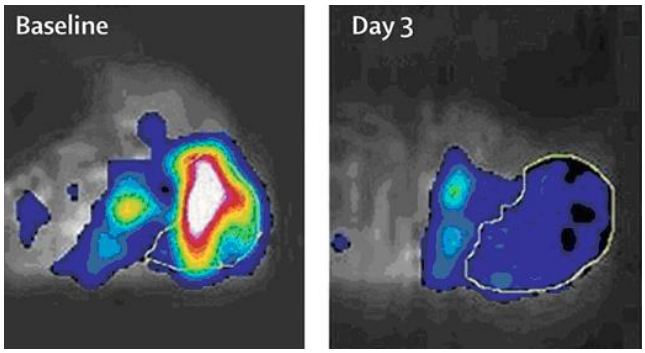

Figure 3.1.: ASL MRI shows decreased perfusion on day 3. Courtesy of Schor-Bardach et al. (71)

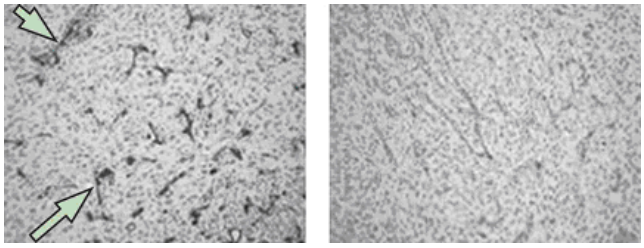

Figure 3.2.: CD34 stain: Decrease of endothelial cells (arrows) on day 3. Courtesy of Schor-Bardach et al . (71)

Under anti-VEGFR-targeted therapy, vessels diminish in quantity, but improve in quality. Pries et al. observed that VEGF-blockade resulted in a temporary and paradoxical "normalization" of tumour vasculature, with selective pruning of poorly formed vessels and a resulting temporary improvement of blood flow and oxygen delivery to the tumour (78). In papillary RCC xenografts in mice treated with sunitinib for 7-18 days, dynamic contrast-enhanced MRI performed after treatment showed increased perfusion and decreased vascular permeability. Histology showed thinning and regularization of tumour vessels (79).

3.3. Multi-target TKIs: targeting other growth factor pathways as well as VEGF-independent angiogenesis

Most of these small TKIs lack specificity and are therefore called multi-target TKIs. These broader substrate specificities may also complicate ascribing their action to purely anti-angiogenic mechanisms, both within the endothelial cell and the tumour cell itself. Sunitinib also blocks rearranged during transcription (RET), c-KIT, fms-related tyrosine kinase 3 (FLT3) and FSC-1R. Pazopanib blocks C-FMS, LCK, ITK, KIT and FGFR1 and -3. Sorafenib blocks RET, FLT3, c-KIT, FGF-receptor-1 (FGFR1), C-RAF as well as wild type and mutant B-RAF. RAF kinase is an important mediator of the RAS/RAF/MEK pathway. Although activating mutations in B-RAF have not been identified in RCC, constitutive activation in the B-RAF pathway (RRAF, MEK, and ERK) has been observed in approximately 50% of tumours (40). As a consequence, the anti-tumour effects of sorafenib may be due to off-target effects on cyclin D1, cyclin B1, survivin and other key regulatory proteins (80).

Moreover, tumour cells can express VEGFR1 and -2, Neuropilin1, PDGFR, FGFR, EGFR and receptors for other angiogenic factors. Hence, anti-VEGFR-targeted therapy could even have a direct cytotoxic effect.

Finally, an immunomodulatory function of TKIs is also possible. As VEGFR1 suppresses dendritic cell function, anti-VEGF-targeted therapy might stimulate the function of dendritic cells and improve the natural anti-tumour immune response.

4. MECHANISMS OF RESISTANCE TO ANTI-ANGIOGENIC DRUGS

4.1 Pharmacokinetic concerns: inadequate target inhibition due to reduced drug levels

All anti-VEGFR-TKIs are administered at a fixed dose. Nevertheless, individual patient characteristics such as differences in absorption, excretion and metabolism as well as drug interactions may influence TKI-serum levels and as a consequence the efficacy of the treatment. Reduced drug plasma concentrations could lead to the development of resistance, or, more accurately, inadequate inhibition of VEGFR-signalling. Moreover, although these findings remain controversial, several publications showed a link between adverse events on TKIs (like hand-foot-syndrome, hypothyroidism and hypertension) and TKI efficacy, an association that could possibly be explained by higher exposure. As a consequence, monitoring drug concentrations to ensure adequate dosing would be of particular interest and could lead to more effective VEGF-pathway blockade through dose adaptations in patients with suboptimal TKI-plasma levels.

After absorption, sunitinib is converted to an equipotent metabolite, SU12662. Since SU12662 has a similar inhibitory profile to sunitinib in preclinical assays, the combination of sunitinib plus SU12662 represents the total active drug in plasma. Both sunitinib and SU12662 are metabolized predominately by cytochrome P450 CYP3A4, and elimination is primarily via the faeces (81). The pharmacokinetics of both compounds are significantly influenced by several covariates including gender, age, and weight. However, in one pharmacokinetic study the magnitude of the predicted changes in exposure minimized the necessity for dose adjustments (82).

Nevertheless, a clear association between sunitinib plasma levels and outcome has been observed: patients with the highest exposure to sunitinib displayed greater tumour size decreases and longer time-to-tumour-progression (TTP) and OS. There was a significant relationship between exposure and the probability of achieving a PR or complete response (CR) in mRCC patients. Thus, this analysis highlights the importance of maintaining patients on the maximal tolerated dose as long as possible and striving to avoid unscheduled dosing interruptions or titration during treatment.

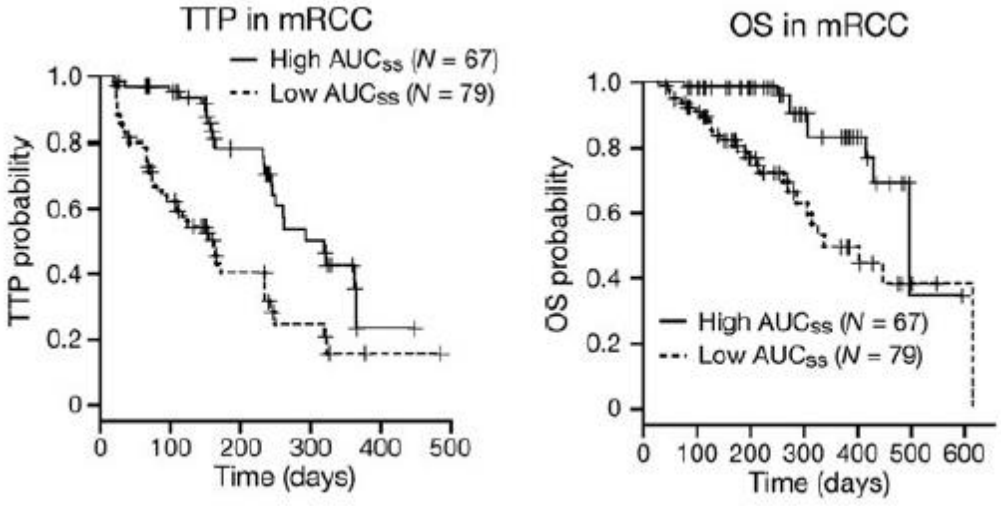

Fig. 5.1.: Relationship between average daily exposure (mean daily area under the curve (AUC) at steady state, AUC_{ss}) to sunitinib and TTP/OS. Courtesy of Houk et al. (83)

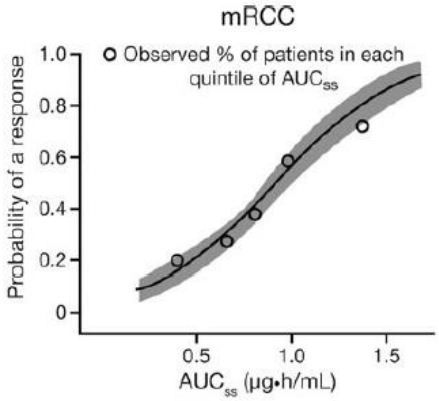

Fig. 5.2.: Probability of a partial or complete response (by RECIST criteria) *versus* average daily exposure (mean daily AUC at steady state, AUC_{ss}) to sunitinib. Lines represent model prediction and shaded area represents 95% confidence interval. Courtesy of Houk et al. (83)

Moreover, in this study, the variability in clearance produced similar exposure ranges across the doses. The starting dose in all patients was 50 mg/day. This dose was adapted during treatment in function of tolerance. Final doses ranged from 25 to 75 mg/day. Nevertheless, the final plasma levels were nearly identical in all patients (83).

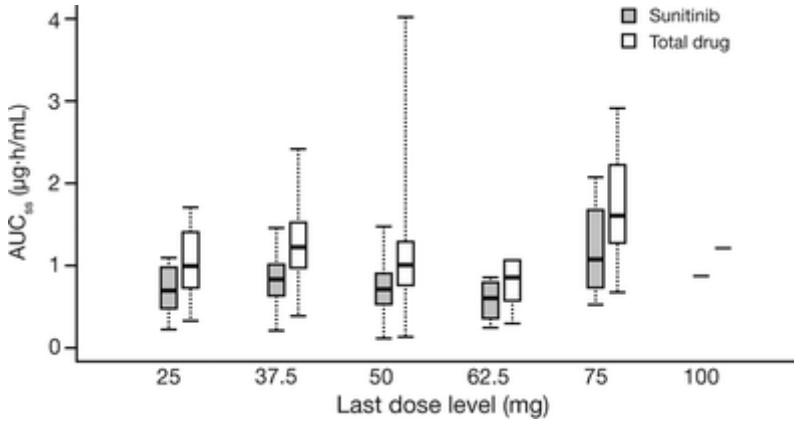

Fig. 6.: Average daily exposure (mean daily AUC at steady state, AUC_{ss}) to sunitinib and total drug (sunitinib + its active metabolite SU12662) calculated at each patient's final dose level. Courtesy of Houk et al. (83)

Moreover, in a randomized phase II study, 213 patients were treated with axitinib 5 mg twice daily for four weeks, after which eligible patients (who did not experience grade 3/4 axitinib related toxicities and no hypertension) were randomly assigned further treatment with axitinib dose titration (stepwise from 5 to 7 to 10 mg twice a day based on tolerability) or axitinib with placebo dose titration. Patients who did not meet eligibility criteria were treated in a separate arm (axitinib ≤5 mg twice daily without dose titration). Among all patients, the mPFS was 14.5 months and RR was 48%. A higher axitinib exposure was associated with a higher RR (59 versus 40%) and an improvement in PFS (14 versus 11 months) (91).

4.2. The evidence of the angiogenic escape under VEGFR-blockade

New imaging techniques support the idea that revascularisation is linked to resistance to VEGF-blocking therapy, a concept called “angiogenic escape”. After rapid lowering of tumour blood flow and normalization of the blood vessels at onset of therapy, a subsequent increase of the number of blood vessels and of the blood flow can announce progression.

In RCC xenograft models, the development of resistance is consistently preceded by restoration of blood flow, as determined by perfusion scanning and by histology showing infiltration of the necrotic tumour remnant by endothelial cells (71).

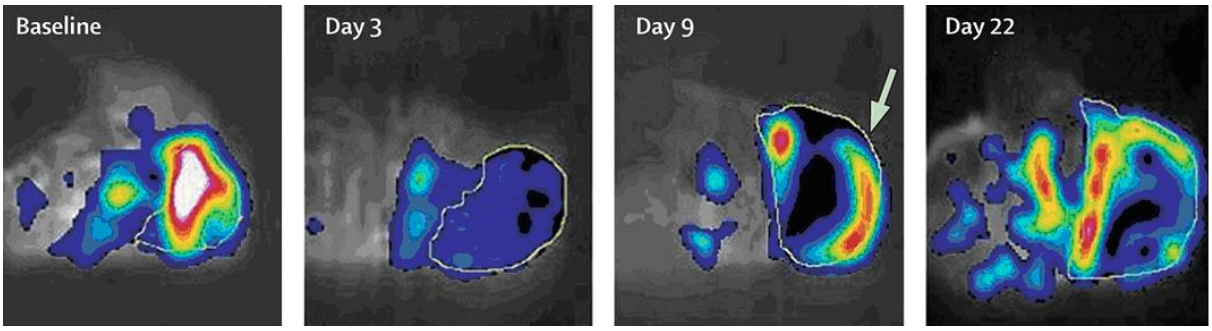

Figure 7.1.: ASL MRI shows decreased signal on day 3 with return of peripheral signal from Day 9 to 22. Courtesy of Schor-Bardach et al. (71)

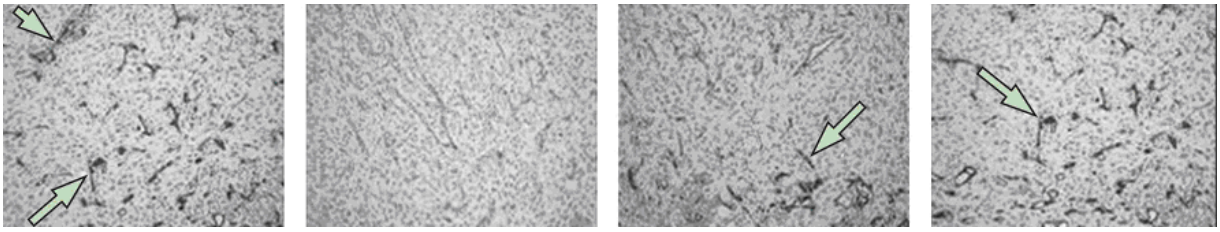

Figure 7.2.: CD34 stain: Viability and vessels correlated to new ASL MRI signal on day 9 and 22. Arrows show endothelial cells. Courtesy of Schor-Bardach et al. (71)

In mRCC patients treated with vatalanib, an experimental anti-VEGFR-TKI, tumour blood flow after 1 month therapy, as assessed by ASL MRI, was positively correlated with size changes at 4 months. Changes in tumour perfusion at 1 month (increase or decrease) were a better predictor of PFS than RECIST-defined measurements of tumour size (84).

These observations suggest that persistence or re-emergence of vasculature is relevant to resistance to anti-VEGFR-TKIs. This re-establishment of vasculature can be less dependent, but not necessarily independent, of VEGF.

4.3. Inadequate target inhibition due to enhancement of the HIF-VEGF-pro-angiogenic pathway

During treatment with anti-VEGFR-TKIs, tumoral hypoxia could increase leading to increase of HIF and subsequent enhanced transcription of the targets of HIF, among them genes involved in the VEGF-dependent angiogenesis pathway. In case of elevation of the VEGF-levels, the VEGF-blockade could become insufficient leading to angiogenic escape. Several experiences seem to strengthen this hypothesis.

An up-regulation of circulating VEGF-levels during VEGFR-blockade with sorafenib was observed in the sorafenib RCC registration trial. Patients treated with placebo did not have an increase in circulating VEGF-levels (21, 85). Similarly, an increase in VEGF-levels in patients treated with sunitinib was found (86). These increasing VEGF-levels under anti-VEGFR-TKI treatment could also explain some cases of rapid progression once anti-angiogenic therapy is stopped with the rapid formation of new vessels (87).

The persistent, albeit less pronounced, efficacy of VEGF-pathway blockers after the development of resistance to an initial VEGFR-blockade, suggest that a component of the tumour remains dependent on VEGF and that this resistance can be countered by an improved VEGF-blockade. The level of tumour susceptibility might depend on features of previous VEGF-targeting drug exposure, including the relative potency of each agent against VEGFR.

The efficacy of sorafenib in patients progressing after sunitinib was assessed in a phase II study and objective responses to sorafenib were observed in 5 of 52 patients (10%), with a median TTP (mTTP) of 16 weeks (88).

In a randomized phase II trial with sorafenib, 43 (66%) of 65 patients who progressed on sorafenib 2x400mg/d had their dose escalated to 2x600mg/d. Forty-two percent of patients had a reduction in tumour size, although no objective responses according to RECIST were noted. mPFS was 3.6 months for patients escalated to sorafenib 2x600mg/d (89).

Axitinib, an anti-VEGFR-TKI with high potency to block VEGFR2, was assessed in a phase II trial in patients with mRCC, all of whom were refractory to sorafenib (77%) or sunitinib (23%). Fourteen of 62 patients (23%) achieved a PR. mPFS was 7.4 months and mOS 14 months. Tumour shrinkage was noted in 80% of patients (90). In a phase III trial, axitinib was studied against sorafenib in ccRCC patients who were progressive on one previous therapy line, mostly cytokines (35%) or sunitinib (54%). In patients who received sunitinib in first-line, mPFS was 4.8 months for axitinib (n=194) and 3.4 months for sorafenib (n=194). The RR was 11.3% in patients treated with axitinib and 7.7% in patients treated with sorafenib (20).

4.4. Up-regulation of alternative VEGF-independent angiogenesis

Although there is abundance of HIF-enhanced pro-angiogenic factors besides VEGF- and PDGF-dependent angiogenesis, the VEGF-signalling pathway seems to be early over-expressed in ccRCC pathogenesis. As a consequence, VEGF-blockers could stop the whole angiogenesis in some ccRCC. Nevertheless, in a later disease stage, or in case of an efficient blocking of the VEGF-signalling pathway, the other pro-angiogenic factors can be responsible for further vessel growth.

In late-stage pancreatic islet tumours in mice, unless efficient VEGF-blocking, neo-angiogenesis starts again under influence of other pro-angiogenic factors such as the FGF. VEGFR2-blockade with a neutralising monoclonal rat anti-VEGFR2-antibody induced an initial 50% reduction in tumour size and microvessel density that was inevitably followed by tumour regrowth, despite sustained VEGFR2-dephosphorylation in the tumour

endothelium. In the latter phase, there was tumour progression, re-induction of angiogenesis and reestablishment of the typically dense and aberrant tumour vasculature. The concurrent administration of an adenovirus encoding a soluble form of FGFR2 (which binds many members of the FGF-family) decreased tumour regrowth and revascularisation, implicating members of the FGF-family as the crucial growth factors responsible for VEGF-independent tumour growth in this model (91). In another study, several members of the FGF-family of pro-angiogenic factors were up-regulated during a VEGFR2-blocking treatment (92).

IL8 is an alternative VEGF-independent pro-angiogenic growth factor. IL8 can enhance tumour angiogenesis in a setting in which VEGF production is impaired, and might have a similar role in circumstances in which VEGF is rendered less relevant due to drug-mediated receptor blockade. IL8-mediated angiogenesis was identified as a key compensatory mechanism of resistance to sunitinib in murine models of RCC. An anti-IL8 antibody did not affect tumour growth in xenograft-bearing animals not yet exposed to an anti-VEGFR-TKI, but after development of resistance to sunitinib, the combination of sunitinib and an anti-IL8 antibody efficiently reduced tumour growth. In addition, IL8-expression has been observed to be elevated in RCC tumours from patients refractory to sunitinib treatment (93).

Considerable evidence suggests that the ANG2/Tie2-axis has angiogenic potential that could parallel the VEGF axis. In preclinical studies, inhibition of ANG2 led to suppression of tumour growth (94). ANG2-levels rise in the plasma of patients at the time of resistance to sunitinib. Therefore, ANG2-inhibition could prevent the revascularisation of tumours at the time of resistance.

4.5. Other mechanisms of escape

In most cases, tumoral regrowth is preceded by neo-angiogenesis. In other cases, relapsing metastases do not display contrast enhancement on imaging, thus no important neo-vascularisation. In these cases, tumour regrowth seems to be independent of neo-angiogenesis and could be the consequence of the activation of new pathways inducing cell growth such as EMT.

One concern of anti-VEGF-targeted therapy in ccRCC is that in contrast to c-kit inhibition in bcl-abl mutated chronic myeloid leucemia or GIST, VEGF-blocking agents are not blocking an activating mutation, but acting far downstream the initial dysregulated site, which is the metabolism of HIF. By blocking VEGF- and PDGF-pathways, we are in most cases even not acting directly on the tumoral cells, but only on the endothelial cells and pericytes. The other genes over-expressed by HIF and involved in tumour growth and invasiveness, are not directly blocked by VEGFR- and PDGFR-targeted therapy, unless indirectly through down-regulation of HIF-levels through decrease of tumoral hypoxia.

Several signalling pathways involved in neo-angiogenesis and cell cycle induction can be up-regulated in RCCs leading to enhanced tumour growth. The mTOR-pathway seems to play a central role in this up-regulation. In RCC as well as in other tumour types, the mTOR-pathway is activated, although rarely through activating mutations. Inhibition of the mTOR-pathway through mTOR-inhibitors produces a modest anti-tumoral activity in RCC as well as in pancreatic neuro-endocrine tumours and in breast carcinoma (5, 6).

EMT is a reversible process through which normal cells or cancer cells lose their epithelial characteristics and acquire a mesenchymal phenotype. Through EMT, these cells acquire a spindle shape and exhibit a more aggressive behaviour with enhanced invasiveness, increased metastatic spread and hypoxia-resistance. EMT can be induced by hypoxia and possibly by anti-VEGFR-targeted therapy. In hepatocellular carcinoma, after long-

term exposure to sorafenib, resistant cells changed morphologically into spindle shaped cells and showed loss of cell-to-cell contacts, which are typical features of EMT (95).

Other preclinical data have even raised the possibility that angiogenesis inhibitors might in fact reduce primary tumour growth while at the same time inducing tumour adaptation and progression to greater degrees of tumour invasiveness and metastatic behaviour (96-98). These findings may help to explain the development of resistance to these agents, but their relevance to clinical use of angiogenesis inhibitors in patients with cancer is as yet unknown. Moreover, these effects were observed in pancreatic island tumours in mice and not in RCC (99).

Another mechanism of escaping hypoxia could be new ways of proliferating of the tumours like lymphangitis carcinomatosa. A tumour will normally form spheres, but in lack of oxygen supply, the tumoral cells will rather proliferate along existing blood vessels, where more oxygen is available. Intratumoral heterogeneity could probably also lead to therapeutic resistance, although targeting ubiquitous events will have a global effect on all metastatic sites (64).

4.6. Arguments against intrinsic resistance

There are several reasons to believe that resistance to VEGF-targeted therapy is in most cases not induced by secondary mutations. Mutation in a gene encoding for a key receptor kinase is an unlikely explanation for the development of resistance to VEGF-pathway antagonists. The main target for VEGFR-inhibitors resides on endothelial cells. A mutation conferring treatment resistance would probably need to occur in the tumour endothelium, presumably in the gene encoding VEGFR2. The simultaneous occurrence of such a mutation in the endothelium of each individual tumour metastasis is highly improbable.

RCC xenograft models show that resistance to sorafenib and most of the associated changes in gene expression are reversed by re-implantation of the resistant xenografts into untreated mice (100). This argues against any permanent genetic or epigenetic change in the tumour cells as an underlying mechanism and suggests that resistance, in part, relates to physiological changes in the microenvironment, enabling reestablishment of angiogenesis in the setting of VEGFR-blockade.

Finally, re-exposure to targeted therapy after a period of discontinuation can be effective in a subset of mRCC-patients. The efficacy of anti-VEGFR-TKI re-treatment was evaluated in 36 patients with disease progression after a TKI-everolimus sequence. The RR with TKI re-treatment was 8%, and the clinical benefit rate (PR plus SD) was 75%. mPFS with each component of the TKI-everolimus-TKI sequence was 10.7, 8.9 and 8.2 months, respectively. mOS from the start of everolimus was 29.1 months, which suggests a benefit in using TKI in this setting. A potential bias may have been incorporated when selecting patients who went on to receive an additional anti-VEGFR-TKI after everolimus. The prolonged PFS observed with third-line (or higher) anti-VEGFR-TKIs in this analysis suggests that mRCC may be re-sensitized to anti-VEGFR-TKI therapy after everolimus (101). Other reports have also suggested renewed anti-tumour activity of anti-VEGFR-TKIs after a period of treatment with an alternate mechanism of action or temporary discontinuation (102, 103).

5. PROGNOSTIC AND PREDICTIVE MARKERS IN CLEAR CELL RENAL CELL CARCINOMA TREATED WITH ANTI-VEGFR-TKIs

Before summarizing all described prognostic and predictive markers associated with outcome in the metastatic setting under VEGF-blockade, we give here below an explanation on the differences between prognostic and predictive markers and an overview of prognostic markers after nephrectomy in localized RCC performed with curative intent.

5.1. Prognostic and predictive markers

When studying the outcome in anticancer therapies, it is important to distinguish between prognostic and predictive markers. Prognostic markers tell us something about the aggressiveness and spontaneous course of the disease: they are linked to the natural evolution of the disease. Predictive markers tell us something about the expected efficacy of a therapy, thus the potential impact of therapy on the further course of the disease. They predict if the disease will likely respond to the therapy or not.

Common parameters measuring the evolution of the disease are PFS and OS: indolent tumours have a long PFS and OS, while aggressive tumours have a shorter PFS and OS. The efficacy of a therapy can be described by PFS, OS or the response rate (RR), which is the assessment of the frequency of tumour shrinkage on a given treatment. An efficient therapy should theoretically prolong PFS and OS, and can lead to clinically relevant tumour shrinkage.

As PFS and OS are both affected by the biology of the disease and by therapeutic efficacy, it is not always easy to make a distinction between a predictive and a prognostic marker. An additional difficulty is that one marker can be predictive and prognostic at the same time, and even in the opposite direction. The best known example is HER2/neu-amplification in metastatic breast cancer: patients with HER2/neu positive breast cancer have a more aggressive disease, but they will specifically respond on HER2/neu-targeted therapy. Thus, HER2/neu-amplification is a negative prognostic marker and on the same time a positive predictive marker.

A prognostic marker can potentially influence both PFS and OS in the same way. A predictive marker could have an impact on PFS without any effect on OS, but can influence both, as a longer PFS can predict a longer OS. How to make thus the difference between a prognostic and a predictive marker?

The easiest way to make the difference is to study the same marker on an untreated control group or on patients treated with placebo. If the effect of a marker on the outcome of an active therapy is not seen in the placebo-treated group, then we have found a predictive marker. On the opposite, if the marker has the same impact in both treatment groups, independently of the given treatment, then we are dealing with a prognostic marker. Nevertheless, a placebo-controlled group is not always available. Only in two pivotal RCC studies with anti-VEGFR-targeted therapies, with placebo control, research on predictive markers has been prospectively done: the pivotal trials of sorafenib and pazopanib (Table 3) (4, 21).

Although less reliable, the RR could also give us some indications about the predictive value of a given marker. In fact, a reduction in size of the tumoral mass is rarely part of the natural evolution of the disease. Thus, if the reduction of the tumoral mass by a particular compound is influenced by a marker, this marker has a predictive value. Of note, the RR to RCC treatments in the pre-TKI era did not correlate with survival.

Moreover, markers clearly linked to pharmacokinetics, more in particular absorption, metabolism and excretion of therapeutic compounds, usually should play a predictive role, as they are not linked to tumour biology and malignancy.

5.2. Prognosis of RCC after nephrectomy with curative intent for localized disease

A multitude of prognostic factors predicting relapse after nephrectomy have been described. The anatomic extent of disease is the most consistent factor that influences prognosis. Five year survival rate is 90% in WHO stage I, 75-95% in stage II and 59-70% in stage III tumours. In stage IV tumours, mOS is 16 to 20 months. Histologic grade is an independent factor correlating with survival: five-year survival rates of 89, 65, and 46% for tumours of histologic Fuhrman grade 1, 2, and 3-4, respectively, were reported (13, 104, 105). A poor prognosis is associated with the presence of a sarcomatoid pattern (106).

Negative clinical prognostic signs include the presence of symptoms and/or para-neoplastic syndromes, obesity, a poor performance status, baseline serum lactate deshydrogenase (LDH) levels, baseline corrected calcium levels, baseline haemoglobin and the interval between the initial diagnosis and the start of systemic therapy. The latter five parameters, that are part of the MSKCC score, were analysed in a series of 118 patients who all had developed a recurrence following nephrectomy for localized disease. Patients with none of these risk factors constituted a low-risk group, while those with one or two risk factors had an intermediate risk and those with three or more were at high risk for shortened survival. mOS after nephrectomy for low-, intermediate-, and high-risk groups was 76, 25, and 6 months, respectively (107).

Although none of these factors has an established role independent of stage, some molecular parameters have shown promise as prognostic markers in patients with ccRCC in the post-nephrectomy setting. Lack of B7H1 and B7H4 expression in patients have been strong predictors of OS (108, 109). Low levels of carbonic anhydrase IX (CAIX) expression and high levels of Ki67, detected by IHC, were associated with a significantly worse prognosis (107). Patients with a deletion of chromosome 9p have more aggressive disease at presentation, manifested by significantly larger tumours, higher Fuhrman grade, and an increased frequency of lymph node and distant metastasis. In patients with chromosome 9p deletion and small (<4 cm) solitary renal masses, there was an increased risk of disease recurrence (110). m-ccRCC patients with chromosome 8q amplifications (111) or chromosome 14q loss (112) had poor survival. In ccRCC, higher VEGF expression correlated with higher tumour size, higher Fuhrman grade, tumour necrosis, higher tumour stage, RCC progression rate and lower RCC-specific survival (113, 114).

VHL-disease-associated ccRCCs seem to grow more slowly and are associated with an overall better prognosis than sporadic ccRCCs (115, 116). Sporadic ccRCCs that lack functional VHL-protein might, therefore, be expected to have a better prognosis than sporadic ccRCCs resulting from VHL-independent mechanisms of pathogenesis and tumorigenesis. However, although the results of some studies seem to support this hypothesis (32, 117), others have found no association between the presence or absence of VHL-alterations and prognosis or adverse clinical and pathological features (36, 115, 118-122). Results of a large case-control study in patients with RCC showed that the presence of *VHL* nonsense mutations was strongly associated with increased tumour grade and lymph-node involvement, and that such mutations were particularly prevalent in patients with metastatic disease (36).

Multiple models have been developed to integrate the information from anatomic staging with histopathology and clinical prognostic parameters. The most widely studied prognostic model has been the UCLA integrated staging system which incorporates the Eastern Cooperative Oncology Group Performance Status (ECOG PS) and Fuhrman's histologic grade (1 through 4) into the TNM anatomic staging system. Using these variables, five distinct prognostic categories were identified that correlate with post-nephrectomy outcome.

Finally, expression-profile based subgroups of ccRCCs such as those described by Brannon et al. and the TCGA seem also to have a prognostic impact: in both studies, there was an association between the molecular subgroups and post-nephrectomy survival (64, 66). For cancer-specific survival after nephrectomy, Brannon's ccA-subtype was associated with a highly significant survival advantage over ccB-patients (p=0.0002, mOS of 8.6 *versus* 2 years). At 5 years, cancer-specific survival was 56% in ccA-patients and only 29% in ccB-patients. The same impact was observed for OS, with a significantly greater survival for ccA-patients over ccB-patients (p=0.004, mOS of 4.9 *versus* 1.8 years). At 5 years, survival for ccA-patients was 48% but only 23% for ccB-patients.

5.3. Clinical-biological markers and prognostic scoring systems

5.3.1. Routinely available clinical and biochemical markers

Several routinely available clinical and biological markers linked to PFS and OS have been described and combined in prognostic scoring systems.

Five factors predicting shortened survival were identified in a series of 670 patients with advanced RCC treated with immunotherapy at the MSKCC (table 4): A Karnofsky performance status (KPS) of <80, serum LDH-level >1.5 times the upper limit of normal, corrected serum calcium >10 mg/dL (2.5 mmol/L), haemoglobin concentration less than the lower limit of normal and absence of nephrectomy (no disease-free interval). Patients with none of these risk factors (good prognosis group) *versus* those with one or two (intermediate prognosis group) *versus* those with three or more risk factors (poor risk group) had significantly higher survival rates at one (71 *versus* 42 and 12%, respectively) and three years (31 *versus* 7 and 0%, respectively) (27). A follow-up analysis with 463 patients who were treated with IFN-alpha identified an interval of less than one year from initial diagnosis to the start of IFN-alpha therapy as an additional indicator of a poor prognosis (8). This parameter replaced the parameter 'absence of nephrectomy' in the score. In this report, the mOS for patients with good, intermediate or poor risk was 30, 14, and 5 months, respectively.

PROGNOSTIC FACTORS	ONE POINT IF	TOTAL SCORE
Karnofsky PS	< 80	0: favourable prognosis 1-2: intermediate prognosis 3-5: poor prognosis
Interval between diagnosis and start of systemic treatment	< 12 months (or no nephrectomy)	
Haemoglobin	< lower limit of normal 11.5 g/dl in women 13.0 g/dl in men	
LDH	> 1.5 upper limit of normal	
Corrected calcium	> 10.00 mg/dl	

Table 4: MSKCC prognostic factors for OS in mRCC (8)

The MSKCC prognostic model was validated and extended in a series of 353 previously untreated patients with mRCC at the Cleveland Clinic (123). Two additional significant negative prognostic factors were identified: prior radiotherapy and the presence of more than one site of metastatic disease. Patients with favourable (no more than one poor prognostic factor), intermediate (two prognostic factors), or poor risk (more than two) disease had a mOS of 26, 14, and 7 months, respectively.

In a series of 375 patients who received sunitinib in the registration trial, high corrected calcium levels, a high number of metastatic sites (more than one), the presence of liver metastases, baseline thrombocytosis, a shorter time from diagnosis to treatment (less than one year) and higher baseline serum LDH-levels were associated with a shorter PFS. Based on this results, a nomogram for predicting 12-months PFS was established (124).

Patil et al. checked the different parameters of the MSKCC-score prognostic for OS and developed during the era of immunotherapy on the 375 patients included in the sunitinib pivotal trial. Multivariate analysis identified 5 factors as significant predictors for PFS (serum LDH-level, presence of ≥2 metastatic sites, absence of prior nephrectomy, ECOG PS and baseline platelet count) and 6 independent factors for OS (serum LDH-level, corrected serum calcium level, time from diagnosis to treatment, haemoglobin level, ECOG PS and presence of bone metastasis). The authors concluded that the MSKCC model for OS is applicable in the era of targeted therapy but added a new independent factor for OS: the presence of bone metastases (125).

The IMDC developed a scoring system based on 645 mRCC patients (clear cell and non-clear cell) treated with three anti-VEGF-targeted drugs including sunitinib (n=396), sorafenib (n=200) or bevacizumab (plus IFN-alpha) (n=49) as first-line anti-VEGF-targeted therapy. Four of the 5 MSKCC criteria for OS were confirmed as independent predictors of short survival (baseline haemoglobin, baseline corrected calcium, baseline KPS<80, interval between diagnosis and start of systemic treatment less than one year), and two new factors were added: baseline neutrophil count and platelet counts higher than the upper limit of normal. Based on these criteria, patients were divided into favourable risk (no adverse prognostic factors), intermediate risk (one or two adverse prognostic factors) or poor risk (three or more adverse prognostic factors) categories. mOS for good-risk patients had not been reached and was 27 and 9 months, respectively, for the intermediate and poor-risk groups (9). In 2013, Heng et al. validated this score on an independent series of 1.028 patients (126).

PROGNOSTIC FACTORS	ONE POINT IF	TOTAL SCORE
Karnofsky PS	< 80	0: favourable prognosis 1-2: intermediate prognosis 3-6: poor prognosis
Interval between diagnosis and start of systemic treatment	< 12 months (or no nephrectomy)	
Haemoglobin	< lower limit of reference 11.5 g/dl in women 13.0 g/dl in men	
Baseline neutrophil count	> 4.500/mm³	
Corrected calcium	> 10.00 mg/dl	
Baseline platelet counts	> 400.000/mm³	

Table 5: IMDC prognostic score for OS in mRCC (9)

Most of these clinical and biochemical markers are also valid in placebo-treated patients, they have a prognostic but not a predictive value (127). They are almost all the reflection of the clinical stage of the malignancy and of the impact of the disease on the general state of the patient.

5.3.2. Tumour burden

In a series of 124 m-ccRCC patients treated with sorafenib or sunitinib (66%) or placebo (34%), tumour burden was directly related to PFS and OS and these associations remained significant after adjusting for MSKCC risk class and treatment. Each 1-cm increase in tumour burden increased the risk of progression by 4.5% and the risk of death by 5%. When adjusting for age, gender, MSKCC risk group (high- *versus* low-/intermediate-risk group), active therapy (yes *versus* no) and type of study, the increase in tumour burden remained associated with a higher risk of progression. In multivariable analysis adjusted for MSKCC prognostic score groups, the tumour burden remained an independent prognostic factor for death. The mOS value for tumour burden above the median was 16.4 months compared with 27.4 months for patients with tumour burden below the median; the differences were maintained when adjusted for treatment (128). In another study on 69 m-ccRCCs treated with sunitinib, tumour burden was found to be a significant predictor of PFS and OS (129).

Other arguments in favour of the impact of tumour burden come from the impact of nephrectomy on outcome. In the era of immunotherapy, nephrectomy, even in locally advanced and metastatic setting, was proved to be a factor of good prognosis (27). A reduction of the tumour load could decrease immunosuppressive effects of the tumour and decrease the amount of tumour stimulating cytokines released by the tumour.

5.3.3. Side effects under anti-VEGFR-TKIs associated with efficacy

The incidence of TKI-induced side effects like hand-foot-syndrome, hypothyroidism and hypertension were in some studies associated with efficacy. This association can be explained by the parallelism between toxicity and efficacy in function of the plasma levels of the active compounds, but biases are easily possible. The longer a patient stays on treatment, the higher the probability that he will develop side effects. Thus, the incidence of this specific side effects should always be related to duration of therapy. The most solid data are on the association between the development of hypertension with anti-VEGFR-TKIs and outcome. In a series of 111 mRCC patients

treated with sunitinib, the development of hypertension during sunitinib treatment was a positive predictive factor associated with a significantly longer PFS and OS. A disadvantage of this approach is that these adverse events only appear once the treatment is started. Thus, they cannot predict efficacy of a planned treatment (130).

5.3.4. Site of metastasis

As anti-VEGFR-targeted therapy is also acting on the tumour microenvironment, efficacy could be very different depending on the site of metastasis. In the MSKCC nomogram predicting PFS, the presence of liver metastases was associated with a shorter PFS (124). Pancreatic metastases seem to be associated with a better OS (131).

5.4. Histology

The presence of sarcomatoid dedifferentiation in the primary tumour has a negative impact on outcome on anti-VEGFR-TKIs in the metastatic setting. In a series of 43 sarcomatoid mRCC treated with anti-VEGF-targeted therapy, PR was observed in 19%, stable disease (SD) in 49% and early progressive disease (PD) in 33% of patients. mPFS and OS were 5.3 and 11.8 months, respectively. PRs were confined to patients with tumours with <20% of sarcomatoid elements. Early PD was observed in only 22% of these patients, while early PD presented in 56% of the patients who had >20% of sarcomatoid elements in their primary tumours. The differences in PFS (6.8 *versus* 4.3 months) and OS (14.9 *versus* 8.6 months) favoured the group that had <20% of sarcomatoid elements, but these differences were not statistically significant (132).

The second largest series of mRCC patients with sarcomatoid dedifferentiation treated with anti-VEGF-targeted therapy involved 32 patients treated with first-line sunitinib (29 patients) or sorafenib (3 patients). Of the patients treated with sunitinib, 14% had a PR, 59% had SD and 28% PD as best response. Five patients received sunitinib as second-line therapy and one of these patients achieved a CR. mPFS and mOS under sunitinib were 4.4 and 10 months, respectively (133). Unfortunately, Golshayan and Molina did not have a control group of patients without sarcomatoid dedifferentiation.

5.5. Non-routine serum markers

5.5.1. Baseline VEGF-levels

Unless the important role of VEGF in early neo-angiogenesis, it has been difficult to determine the prognostic and predictive role of serum VEGF-levels. Contradicting results may be due to problems of VEGF-detection. Elevated baseline serum VEGF-levels seem to be a negative prognostic marker in RCC. In mRCC patients treated with immunotherapy, higher baseline serum VEGF-levels predicted poorer PFS and OS (134). However, in other studies, this association was not confirmed (135, 136). In patients treated with sorafenib or placebo in the pivotal phase III trial, higher baseline VEGF-levels were associated with poorer OS in both treatment arms (2, 85). Increased baseline VEGF-level was also found to be associated with decreased survival in sunitinib treated patients. Patients with higher baseline VEGF-levels had a higher probability of disease progression (137).

On top of a negative prognostic value, higher VEGF-levels might have an additional positive predictive value. In the pazopanib pivotal trial, when comparing OS in patients treated with placebo and pazopanib and with low and high baseline VEGF-levels, higher VEGF-levels were a negative prognostic markers for OS in both treatment groups, but associated with a larger relative OS benefit when treated with pazopanib (127). In the placebo group of the sorafenib pivotal trial, high baseline VEGF patients had a shorter PFS than patients with low baseline VEGF, reflecting an aggressive tumour and thus indicating that high baseline VEGF-levels have a negative prognostic value. Nevertheless, patients with high baseline VEGF-levels derived more benefit from sorafenib relative to placebo than those who had low baseline VEGF-levels, although the mPFS for sorafenib treatment in both groups was the same (2).

5.5.2. Evolution of VEGF, sVEGFR2 and sVEGFR3 during therapy

In a biomarker analysis from the sunitinib phase II trial in cytokine-refractory RCC patients, significantly larger changes in VEGF- (increased during treatment) and soluble VEGFR2- and VEGFR3-levels (both decreased during treatment) were seen in patients who yielded a PR to treatment than in those who had SD or PD (86, 138). For pazopanib, a decrease in serum VEGFR2-levels during therapy was also linked to improved response and PFS (139). In the sorafenib registration trial, serum VEGFR2-levels declined under treatment with sorafenib, but not in the group treated with placebo. Nevertheless, the authors did not study the link between the decrease of the biomarker and treatment outcome (85).

5.5.3. Baseline serum levels of IL-6 and IL-8

IL6 is a potent inducer of an inflammatory reaction and IL8 an alternative VEGF-independent pro-angiogenic factor. In a series of 225 pazopanib treated mRCC patients, higher baseline serum IL6- or IL8-levels were associated with a more pronounced tumour shrinkage (IL8), shorter PFS (IL6 and IL8) and OS (IL6). These associations were also found in 118 placebo-treated patients, indicating the prognostic value of these cytokines. Nevertheless, higher baseline IL6- or IL8-levels were associated with a larger relative benefit when treated with pazopanib, suggesting that on top of a negative prognostic value, higher baseline IL6- or IL8-levels might also have a positive predictive value (127).

5.6. Molecular markers

5.6.1. *VHL*-mutation and hyper-methylation

Given the important role of the loss of VHL-function in the molecular pathogenesis of RCC and in VEGF-dependent angiogenesis, an attractive hypothesis was that *VHL*-mutation or *VHL*-hyper-methylation would render a tumour more VEGF-dependent and that *VHL*-wild type tumours would be rather resistant to anti-angiogenics. Nevertheless, this hypothesis could never be confirmed. In a series of 43 m-ccRCC patients, 26 (60%) had *VHL*-mutations or promoter hyper-methylation and 17 (40%) were *VHL*-wild type. The RR on anti-angiogenic drugs (bevacizumab plus IFN-alpha, sunitinib or axitinib) was 48% *versus* 35% with a mTTP of 10.8 *versus* 5.5 months

(p=0.26), respectively. In 15 patients with VHL-methylation or VHL-mutation predicted to truncate or shift the VHL reading frame, mTTP was 13.3 *versus* 7.4 months (p=0.06) in patients with none of these features (140).

In a series of 123 m-ccRCC patients treated with sunitinib (51%), sorafenib (23%), axitinib (12%) or bevacizumab (14%), the overall frequency of *VHL*-mutations was 49%, and 78% of these mutations were predicted to result in the loss of function of the VHL-protein (frame shift, nonsense, splice site, and in-frame deletions or insertions). Ten percent of patients had *VHL*-promoter hyper-methylation. The RR was not significantly different in patients with inactivated (mutated or methylated) *VHL* compared with those who had wild-type *VHL* (41% *versus* 31%, p=0.34). Patients with loss of function mutations had a 51% RR, compared with 31% in wild-type *VHL*-carriers (p-value: 0.04). On multivariate analysis, that included several other important clinical prognostic factors, the presence of a loss of function mutation remained an independent prognostic factor associated with improved response. Nevertheless, PFS was not modified: patients with *VHL*-mutation had a mPFS of 12 months *versus* 9 months for *VHL*-wild-type patients and 11 months for *VHL* hyper-methylated patients (p=0.78). Patients with a *VHL*-loss-of-function-mutation had a mPFS of 13.7 months *versus* 9 months for *VHL*-wild-type patients (p=0.71) (141).

This surprising finding can probably been explained by the fact that other mechanisms beside *VHL*-mutations or promoter hyper-methylation can lead to the dysfunction of VHL-protein or HIF-stabilization. In ccRCCs without VHL-mutation or promoter hyper-methylation, other mechanisms of VHL-impairment or HIF-stabilization are often present. This could explain why it has been difficult to find associations between outcome on anti-VEGFR-TKIs and mutations or promoter-hyper methylation of *VHL*.

5.6.2. Germ-line polymorphisms

Recently, three studies have described associations between single nucleotide polymorphisms (SNPs) in genes involved in sunitinib or pazopanib pharmacokinetics (*ABCB1, NR1/2* and *NR1*/3), sunitinib or pazopanib pharmacodynamics (*PDGFR-alpha*, *VEGFR2* and *VEGFR3*) and VEGF-independent pro-angiogenic pathways (*FGFR2*, *IL8*) and treatment outcome on these anti-VEGFR-TKIs in patients with mRCC. These studies are described in detail in Part 3, 4 and 5 of the Results section (142-144).

5.6.3. Expression levels of hypoxia markers

In a series of 67 m-ccRCC patients treated with sunitinib, the expression of proteins involved in hypoxia pathways was analysed by IHC. Associations were observed between high expression of HIF2-alpha and PDGFR-beta and better sunitinib RECIST objective response. Increased VEGFR3-expression was associated with longer PFS. VEGFR3-overexpression showed a negative correlation with VEGFR3 polymorphism rs307826, a sunitinib resistance predictor. High VEGF-A was associated with short OS and HIF2-alpha with long OS (145).

In a second series of 42 m-ccRCC patients treated with sunitinib, higher HIF1-alpha, VEGFR3 and CD34 (as an assessment for microvessel density) staining were associated with better PFS. Higher VEGFR1 and VEGFR3 staining were associated with better OS. Tumours with characteristics of good prognosis such as low Fuhrman grade and low T-stage (T1 or T2), absence of lymph nodes and absence of synchronic metastases displayed

higher mean expression of CD31, CD34, HIF1-alpha, VEGFR1, -2 and -3 vessel staining as well as PDGFR-alpha and –beta score and intensity (146).

In a third series of 40 m-ccRCC patients, high VEGFR2-levels, as assessed by IHC on the primary nephrectomy specimen, were associated with improved PFS on multivariate analysis when treated with sunitinib (147).

OBJECTIVES OF THE RESEARCH

The objective of this PhD project was to investigate new clinical, biochemical, pathological and molecular markers predictive for response in m-ccRCC patients treated with anti-VEGFR-TKIs. Additionally, we aimed to describe further prognostic markers in m-ccRCCs treated with these therapies.

The specific materials and methods of each subunit of our work are described in the corresponding chapters. For the entire project, we used a newly established clinical database, a tissue collection of paraffin embedded tumour slides, a frozen tumour bank and germ-line DNA samples.

Between 2009 and 2013, the clinical database of patients with mRCC treated with anti-VEGF-targeted therapy included 531 cases, originating from the following centres: University Hospitals Leuven (Leuven, Belgium): 230 patients, Algemeen Ziekenhuis Groeninge (Kortrijk, Belgium): 35 patients, Hôpital Européen Georges Pompidou (Paris, France): 97 patients, Institut Gustave Roussy (Villejuif, France): 67 patients, Centre Hospitalier Universitaire Strasbourg (Strasbourg, France): 17 patients and other institutions in France: 85 patients. Among these cases, 408 were m-ccRCC patients treated in first-line anti-VEGF-targeted therapy with sunitinib. Moreover, the database also contained 55 files of m-ccRCC patients treated in first-line with pazopanib or sorafenib.

Furthermore, a fully annotated RCC tumour bank based at INSERM U674 “Génomique fonctionelle des tumeurs solides”, linked to Université Paris-5 René Descartes, headed by Prof. Dr. Zucman-Rossi, was established. This tumour bank contains 288 fresh frozen samples, including 132 ccRCCs treated first-line in the metastatic setting with sunitinib with complete follow-up of the clinical data. Samples were collected in France (96 samples) and at University Hospitals Leuven (36 samples). Data on response to sunitinib are available. The tumour bank also contains corresponding normal kidney tissue from these patients. For germ-line DNA investigations, we used the frozen normal kidney tissue as well as peripheral blood of those patients in whom frozen non-tumoral tissue was not available.

Most of the patients included in this research work were treated in first-line with sunitinib, fewer patients with pazopanib or sorafenib. As the therapeutic efficacy of anti-VEGFR-TKIs was an important endpoint of our study, only RCCs with clear cell histology were included. In study parts where the efficacy of bone targeted therapies or toxicity of the VEGFR-TKIs and pharmacokinetic aspects of sunitinib were studied, some patients with non-ccRCC were included. Unfortunately, no samples or clinical data from placebo treated patients were available for our project.

RESULTS

PART 1: NEGATIVE IMPACT OF BONE METASTASIS ON OUTCOME IN CLEAR CELL RENAL CELL CARCINOMA TREATED WITH SUNITINIB

PART 2: CONCOMITANT ORAL TYROSINE KINASE INHIBITORS AND BISPHOSPHONATES IN ADVANCED RENAL CELL CARCINOMA WITH BONE METASTASES

PART 3: SINGLE NUCLEOTIDE POLYMORPHISMS ASSOCIATED WITH OUTCOME IN METASTATIC RENAL CELL CARCINOMA TREATED WITH SUNITINIB

PART 4: EFFLUX PUMP *ABCB1* SINGLE NUCLEOTIDE POLYMORPHISMS AND DOSE REDUCTIONS IN PATIENTS WITH METASTATIC RENAL CELL CARCINOMA TREATED WITH SUNITINIB

EFFLUX PUMP *ABCB1* SINGLE NUCLEOTIDE POLYMORPHISMS AND DOSE REDUCTIONS IN PATIENTS WITH METASTATIC RENAL CELL CARCINOMA TREATED WITH SUNITINIB

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ABSTRACT

Background: There is growing evidence that sunitinib plasma levels have an impact on treatment outcome in patients with metastatic renal cell carcinoma (mRCC). We studied the impact of single nucleotide polymorphisms (SNPs) in genes involved in sunitinib pharmacokinetics, and additionally, sunitinib pharmacodynamics on dose reductions of the tyrosine kinase inhibitor.

Methods: We retrospectively analysed normal DNA retrieved from mRCC patients receiving sunitinib as first-line therapy. We genotyped 11 key SNPs, respectively in *ABCB1, NR1/2, NR1/3* and *CYP3A5,* involved in sunitinib pharmacokinetics as well as *VEGFR1* and *VEGFR3*, which have been suggested as regulators of sunitinib pharmacodynamics. Association between these SNPs and time-to-dose-reduction (TTDR) was studied by Cox regression.

Results: We identified 96 patients who were treated with sunitinib and from whom germ-line DNA and data on dose reductions were available. We observed an increased TTDR in patients carrying the TT-genotype in *ABCB1* rs1125803 compared to patients with CC- or CT-genotypes (19 *versus* 7 cycles; p=0.031 on univariate analysis and p=0.012 on multivariate analysis) and an increased TTDR in patients carrying the TT/TA-variant in *ABCB1* rs2032582 compared to patients with the GG- or GT/GA-variant (19 *versus* 7 cycles; p=0.046 on univariate analysis and p=0.024 on multivariate analysis).

Conclusion: mRCC patients carrying the rs1128503 TT-variant or the TT/TA-variant in rs2032582 in *ABCB1*, which encodes for an efflux pump, do require less dose reductions due to adverse events compared to patients with the wild type or heterozygote variants in these genes.

KEYWORDS: renal cell carcinoma, sunitinib, *ABCB1* efflux pump, single nucleotide polymorphisms, dose reductions

INTRODUCTION

Inactivation of the von Hippel–Lindau (*VHL*) tumour suppressor gene is the most frequent molecular alteration in clear cell RCC. Inactivated *VHL* leads to elevated protein levels of hypoxia-induced factor-α which upregulates the vascular endothelial growth factor (*VEGF*) and platelet-derived growth factor (*PDGF*) pro-angiogenic signaling pathways. Targeted therapies directed against the *VEGF*- and *PDGF*-receptor have significantly improved the outcome of patients with mRCC. Sunitinib malate is an orally administered tyrosine kinase receptor inhibitor (TKI) that targets *VEGF* and *PDGF* receptors, KIT, FLT-3, colony stimulating factor-1 receptor, and RET. In a randomized controlled trial sunitinib significantly prolonged progression-free-survival (PFS) (11 *versus* 5 months, p<0.001) as compared to interferon alpha [1]. Median overall survival (OS) was 26.4 and 21.8 months, respectively (p=0.051) [2]. Sunitinib is a current standard treatment option in mRCC, but other anti-*VEGFR* and anti-*PDGFR*-targeted TKIs like sorafenib, pazopanib and axitinib are also used in certain clinical settings.

Although 50% of RCC patients receiving sunitinib experience an objective response and 43% achieve disease stabilization, 7% will experience progressive disease (PD) at first evaluation probably due to intrinsic resistance or due to other factors [2]. Moreover, even patients with an initial clinical benefit will finally progress due to acquired resistance or for other reasons. Although different mechanisms of primary and secondary resistance have been proposed, reliable biomarkers predictive of sunitinib sensitivity or primary/secondary resistance are still lacking [3].

Sunitinib plasma levels are not dosed in clinical routine, even though it is known that sunitinib plasma levels might impact efficacy of sunitinib treatment in mRCC. A population pharmacokinetic analysis of sunitinib and its primary active metabolite, SU12662, found that the pharmacokinetics of both compounds were significantly influenced by several covariates including gender, age, and weight; however, the magnitude of the predicted changes in exposure minimized the necessity for dose adjustments [4]. A meta-analysis of pharmacokinetic data from 443 patients treated with sunitinib showed that higher plasma levels of sunitinib and its active metabolite SU12662 were associated with prolonged time-to-tumour-progression (TTP) and OS [5]. Other studies have shown that the occurrence of adverse events, in particular hypertension, is possibly linked to improved treatment outcome [6]. Finally, several studies in RCC have shown associations between polymorphisms in genes linked to sunitinib pharmacokinetics and outcome on sunitinib [7-10] or pazopanib [11-12].

The main objective of the present study was to analyse the impact of SNPs in selected genes potentially linked to sunitinib pharmacokinetics (*ABCB1, NR1/2, NR1/3* and *CYP3A5*) and the occurrence of dose reductions during treatment. Additionally, we analysed the impact of SNPs in two genes encoding sunitinib targets (*VEGFR1* and *VEGFR3*), linked to sunitinib efficacy, and the occurrence of dose reductions.

MATERIALS AND METHODS

For the purpose of this retrospective study, germ-line DNA samples were collected from the “CIT-rein” kidney tumor bank and from patients treated at the University Hospitals Leuven. The French-Belgian multicentric “CIT-rein” kidney tumor bank contains more than 250 frozen pathologically confirmed RCC tumor samples collected at 20 academic hospitals. In the “CIT-rein” kidney tumor bank, we selected the samples of patients treated in first-line with sunitinib at a starting dose of 50 mg/day 4 weeks on, 2 weeks off and of whom frozen normal kidney tissue as well as data on dose reductions were available. In order to extend the series, we sampled peripheral blood in all the RCC patients treated at the University Hospitals Leuven from July 2011 till December 2012 applying the inclusion criteria. Eligible patients could have received cytokines as systemic treatment for kidney tumors, but they could not have received any other TKI or mammalian target of rapamycin (mTOR) inhibitor before starting sunitinib.

Dose reduction policy and timing of clinical radiological assessments were left to the discretion of the attending doctors in accordance with current local practice guidelines. Usually, whenever necessary for tolerance issues, in a first step, the dose is reduced to 37.5 mg/day and, if necessary, in a second step to 25 mg/day. In some patients, sunitinib is definitively stopped for tolerance issues.

The endpoint of this study was time-to-dose-reduction (TTDR), calculated as the time between the start of sunitinib and the occurrence of a dose reduction to 37.5 mg/day or of definitive stop of sunitinib for tolerance issues. Therefore, in this study, the SNPs were primarily evaluated as toxicity-related markers, although it was also foreseen to check our previous findings on associations between these SNPs and outcome in this patient series in order to show the inverse correlation between TTDR and outcome. If a patient’s regimen was switched from 50 mg/day to 37.5 mg/day continuously because of flare-up during the two weeks off sunitinib, this was not considered as a dose reduction for adverse event and the censing was closed at the moment of dose adaptation.

For the statistical analysis, the genotypes were combined as much as possible, as it was done in the original publications. Details and exceptions to this rule are documented in the legend of table 4.

For those SNPs that were significantly associated with TTDR, we also analyzed their association with PFS and OS. For this efficacy analysis, only patients with clear cell RCC were considered as previous publications on associations between polymorphisms and efficacy were only reported in clear cell RCCs. Moreover, for the efficacy analysis, all the patients had to complete at least one cycle of sunitinib and had to reach at least the first evaluation by CT scan. Response evaluation was done by RECIST in most of the cases.

The protocol was approved by the medical ethics review boards of all participating institutions, and signed consent was obtained from all patients. In some cases, we used frozen biologic material from patients who had already died and for whom a general positive advice for the utilization of remaining tissue was foreseen by the institutional board.

SNPs with potential relevance for sunitinib dose reductions were selected from the literature (Table 1A and 1B). In particular, we included SNPs in genes linked to sunitinib or pazopanib pharmacokinetics or pharmacodynamics associated with efficacy and/or dose reductions in previous publications with sunitinib [6][7][8][9] or pazopanib [10][11].

DNA was isolated from fresh frozen normal kidney tissue sampled in the nephrectomy specimen using the Qiaquick extraction kit (Qiagen, Valencia, CA, USA) and quantified by fluorometry (Fluoroskan Thermo

Labsystems, Cergy-Pontoise, France). DNA was isolated from peripheral blood with the Qiagen DNA kit (Qiagen, Valencia, CA, USA) and the final DNA concentration was quantified with Nanodrop (Nanodrop, Wilmington, USA). High-throughput SNP genotyping was performed using the Sequenom MassArray platform [12]. Investigators blinded for the clinical data performed the genotyping analysis. Overall, the 11 selected SNPs were successfully genotyped with success rates ≥92% for each SNP and an overall average success rate of 98%. For most of the SNPs, genotypes were analyzed in the same way as they were described in the original reports (i.e., according to dominant, recessive or co-dominant genetic models or in the context of a specific haplotype). Details are given in table 4.

Clinical data were collected at 12 different sites in France (11) and Belgium (1). TTDR, PFS and OS were calculated by Cox regression. Based on the data of Houk et al [4], we considered that gender, age and general shape of the patient as reflected by his IMDC prognostic score could influence tolerance and as a consequence TTDR. These factors were tested in univariate analysis, except patient weight which was not available. Any parameter related to TTDR in univariate analysis by Kaplan-Meier with a p-value <0.2 was included in the multivariate model (Cox regression). Without correction for multiple testing, results with a p-value of <0.05 were considered as significant. However, correction for multiple testing by Bonferroni, taking into account the fact that the correlation with 11 SNPs was analyzed, indicated a p-value of <0.005 as the threshold for significance. Statistical analyses were conducted using GraphPad Prism 5 (GraphPad Software, La Jolla, UCLA) and XLSTAT software (Addinsoft, Paris, France).

RESULTS

We used tissue and clinical information from 96 patients who started sunitinib between November 2005 and November 2012 and closed the follow-up database in June 2013. For 72 patients, frozen normal kidney samples from the “CIT-rein” kidney tumor bank were used and for 24 additional patients treated in Leuven, peripheral blood was used. The data of 81 of these patients were used in a previous publication on the impact of *ABCB1* polymorphisms on outcome by our own group [8]. Table 2 shows the clinical characteristics of these patients. Mean age at diagnosis was 59 years (range 25-84). The majority of patients (>95%) were of Caucasian origin. According to the International Metastatic Renal Cell Carcinoma Database Consortium (IMDC) prognostic criteria [13], 14% of patients were categorized into the favorable risk group, 59% had intermediate and 27% poor risk.

Fourty-nine out of 96 patients (51%) required dose reductions or definitive stop of sunitinib after a median TTDR of 5 cycles (46 dose reduction and 3 treatment withdrawal). Median TTDR in all patients, i.e., in those undergoing dose reduction and those not undergoing dose reduction, was 9 cycles. The most frequent reason for dose reduction were hand foot skin reactions (17 patients), followed by diarrhea (14 patients), fatigue (9 patients), arterial hypertension (5 patients) and thrombocytopenia (5 patients). Less common reasons for dose reductions were anorexia, cardiotoxicity, mucositis, nausea and neutropenia. At the time of analysis, 71 (74%) patients had progressed and 59 (61%) had died. The median follow-up was 59 months (range 2 – 89 months) after the start of sunitinib. The median PFS of the whole study population was 15 months and the median OS 29 months. Best response could be evaluated in 91 patients. Seven of 91 (7.7%) patients had a complete response (CR), 33/91 (36.3%) patients a partial response (PR), 37/91 (40.7%) stable disease (SD) and 14/91 (15.4%) progressive disease (PD) as best response.

For each of the 11 genotyped polymorphisms the respective genotypes, allele frequencies and changes at the amino acid level are described in Table 3. The observed allele frequencies for each polymorphism were similar as previously reported in the dbSNP database (dbSNP build 136) or 1000 Genomes Project, except for SNPs rs2276707.

The association between these polymorphisms and TTDR, as assessed by univariate analysis, are reported in Table 4 and displayed in Figures 1-3. We observed increased TTDR in patients carrying the rs1125803 TT-genotype compared to patients carrying the CC- or CT-genotypes in *ABCB1* (19 versus 7 cycles; p=0.031). Likewise, we observed increased TTDR in patients carrying the TT/TA-variant in *ABCB1* rs2032582 (19 versus 7 cycles; p=0.046), but rs1128503 and rs2032582 were in high linkage disequilibrium (r^2=0.984) with each other. We also observed increased TTDR in patients with the TT-genotype in NR1/2 rs2776707 compared to patients with CC- and CT-genotypes (41.5 versus 7 cycles; p=0.027). We could not observe any association between SNPs in *NR1/3, CYP3A5, VEGFR1* and *VEGFR3* and TTDR.

In view of an adjusted p-value and of the multivariate analysis, we checked TTDR in female and male (10 versus 9 months; p=0.13) and in IMDC good and intermediate versus poor risk patients (11 versus 7 months; p=0.054). Age at start of sunitinib (under or above the median age of 61 years) had no influence on TTDR (HR 0.89 (95%CI 0.49-1.60); p=0.69). Unfortunately, patient weight at start of sunitinib therapy was not available in a considerable part of the patients. Taking in to account gender and IMDC, the adjusted p-value is 0.014 for rs1128503 in *ABCB1*, 0.025 for rs2032582 in *ABCB1* and 0.063 for rs2776707 in *NR1*/2.

In a next step, we introduced the other polymorphism in the multivariate analysis. Including gender, IMDC (good and intermediate versus poor), rs1128503 in *ABCB1* and rs2776707 in NR1/2, the p-value for the association between these SNPs and TTDR were 0.012 and 0.058, respectively. With rs2032582 (in *ABCB1* instead of rs1128503) and rs2776707, the p-value for the association between these SNPs and TTDR were 0.024 and 0.060, respectively. Note that rs1128503 and rs2032582 were not included in the same multivariate analysis, because of their high linkage disequilibrium (r^2=0.984).

In a previous publication, we showed the impact of SNP rs1128503 in *ABCB1* on outcome in mRCC treated with sunitinib. In order to show the inverse correlation between TTDR and outcome, we checked the impact of the SNPs associated with dose reductions on outcome. As there is no complete overlap with the previously published series, we report the outcome data of the present patient series. In patients with clear cell histology, we have found a trend to a shorter PFS (11.5 versus 16 months, p=0.078) and a shorter OS (24 versus 34 months, p=0.016) in patients with the TT-genotype compared to patients with the CC- and CT-genotypes in rs1125803 in *ABCB1*, a trend to a shorter PFS (15 versus 18 months, p=0.094) and a shorter OS (26 versus 41 months, p= 0.012) in patients with the TT/TA-genotype compared to patients with the GG- and GA/GT-genotype in rs2032582 in *ABCB1* and a shorter PFS (7 versus 18 months; p=0.011) and a trend to a shorter OS (12 versus 31 months; p=0.14) in patients with the TT-genotype compared to patients with the CC- and CT-genotypes in rs2776707 in *NR1*/2 (Figures 4-5).

DISCUSSION

The main objective of the present study was to analyze the impact of SNPs in selected genes potentially linked to sunitinib pharmacokinetics (*ABCB1, NR1/2, NR1/3* and *CYP3A5*) and the occurrence of dose reductions during treatment. We hypothesized that patients carrying genotypes that reduce absorption of sunitinib or increase metabolism of sunitinib - through lower sunitinib plasma levels and less frequent adverse events - less frequently require dose reductions.

In a series of 96 mRCC patients treated with sunitinib as first-line targeted therapy, we observed an association between SNP rs1128503 in *ABCB1*, rs2032582 in *ABCB1* as well as SNP rs2776707 in *NR1*/2 and the time point of dose reductions during sunitinib treatment, although the latter was not confirmed on multivariate analysis. Our time-to-event approach enabled us to avoid lead-time bias, which could easily have occurred if we would have merely compared the incidence of dose reductions in subgroups with significantly different treatment durations. The impact of these SNPs on PFS and OS was also analyzed on these patients series, showing an inverse correlation between efficacy and dose-reductions. Note that in a previous publication, we had already reported on the association between these SNPs and outcome on a patient series including 81 patients of the present study. In lack of a placebo-treated control group, we cannot define if these SNPs have a prognostic or a predictive value for outcome, although the fact that these genes are involved in sunitinib pharmacokinetics points toward a predictive value.

At the start of therapy, anti-VEGFR-TKIs are generally administered at a fixed dose irrespective of the age, gender, weight or length of the patient. In the case of sunitinib, the starting dose is 50 mg/day for 4 weeks, followed by two weeks off-treatment. Many patients require dose modifications, for instance dose reductions to 37.5 mg/day or even 25 mg/day due to tolerance issues. In the pivotal sunitinib trial, 38% of patients had dose interruptions and 32% had dose adaptations due to toxicity [1]. Remarkably, Houk et al. observed that when doses are lowered to 37.5 mg/day or subsequently even to 25 mg/day due to tolerance issues, or even when the dose of sunitinib is increased to 62.5 mg/day of sunitinib in patients with good tolerance but in need for an increased anti-tumor activity, the plasma levels of sunitinib are remarkably similar in all patients irrespective of dose adaptation [5]. These data suggest that individual patient characteristics that influence TKI absorption, excretion and metabolism may indeed influence TKI plasma levels and as a consequence determine the time and frequency of a dose reduction.

The efflux transporter *ABCB1* (ATP binding cassette member B1, formerly known as P-glycoprotein or MDR1) is expressed in the intestine and liver and involved in the oral absorption and biliary secretion of several anticancer drugs [14]. This transporter may contribute to multidrug resistance in tumors by actively extruding drugs from cancer cells, particularly in RCC [15][16]. As a consequence, expression levels and functionality of these drug transporters, for instance due to polymorphisms, may have important consequences for the efficacy of sunitinib. The most common functional SNPs in *ABCB1* are the synonymous 3435C>T (rs1045642) and 1236C>T (rs1128503) changes and the non-synonymous 2677G>T change (missense A893S/T rs2032582). Functional studies have shown that the haplotype of these three SNPs (rs1046542 – rs1128503 - rs2032582) alters the function of the efflux transporter including its substrate specificity. There are four publications showing an association between rs1128503 in *ABCB1* and treatment outcome on anti-VEGFR-TKIs in mRCC (Table 1) favoring patients with CT- and CC-variants [6][7][8][11]. As a consequence, the TT-genotype could lead to a more

active efflux pump or more affinity of the pump for sunitinib, leading to lower sunitinib plasma levels. Our data suggesting an association between the TT-genotype and a delay in dose reductions supports this hypothesis.

Although Garcia-Donas, on a series of 89 mRCC patients treated with sunitinib, did not observe a higher risk for dose reductions in patients with the *ABCB1* rs1128503 TT-variant or the rs2032582 TT/TA-variant, he observed less hypertension in patients with these variants: HR for the development hypertension was 0.41 (95%CI 0.20-0.81; p=0.011) for the rs1128503 TT-variant and 0.42 (95%CI 0.21-0.84; p=0.014) for the rs2032582 TT/TA-variant [7]. Moreover, on a series of 115 mRCC patients treated with sunitinib, rs2032582 in *ABCB1* was linked to sunitinib plasma concentrations ($p<0.05$) [17].

After absorption, sunitinib is converted to an equipotent metabolite, SU12662 [18]. Both sunitinib and SU12662 are metabolized predominately by cytochrome (CYP) 3A4, and elimination is primarily via the feces. The expression of cytochrome CYP3A4, thought to be the key enzyme for the hepatic biotransformation of sunitinib, is regulated by the ligand-activated nuclear receptors *NR1I2* (pregnane X receptor) and *NR1I3* (constitutive androstane receptor) [19][20]. There is evidence that polymorphisms in *NR1*/2 and *NR1*/3 might be associated with outcome in mRCC treated with anti-VEGFR-TKIs (Table 1). Patients with the TT-genotype in rs2276707 in *NR1/2,* leading to a higher expression of *CYP3A4*, seem to have a shorter PFS and OS. Our findings of an association between the TT-genotype and a decreased delay in dose reductions supports this hypothesis.

We could not find any association between the TT-variant in rs4073054 in NR1/3 and TTDR despite the fact that patients with this variant tend to have a worse outcome. Neither could we find any association between SNP rs776746 in *CYP3A5* and TTDR, although it was shown that the AA- and AG-genotypes were link to improved treatment outcome [6] and to increased need for dose reductions [7].

These findings, when validated, could have interesting clinical applications. In fact, a patient whose disease is primarily or secondarily resistant to sunitinib 50 mg/day, who has few side effects and who has the *ABCB1* rs1128503 TT-variant, the rs2032582 TT-variant or the *NR1*/2 rs2776707 TT-variant, could be a good candidate for a trial with sunitinib dose escalation to 62.5 mg/day or even 75 mg/day. There is evidence for the positive impact of dose escalation of some anti-VEGFR-TKIs on treatment outcome in mRCC. In a randomized phase II trial with sorafenib, dose escalation of sorafenib from 2x400mg/d to 2x600mg/d was foreseen. Forty three (66%) of 65 patients who progressed on sorafenib 2x400mg/d had their dose escalated and 42% of these patients achieved a reduction in tumour size and disease stabilisation. The median PFS was 3.6 months for patients escalated to sorafenib 2x600mg/d. The PFS of escalation of sorafenib was more effective than placebo in this setting [21].

Our study has several potential limitations. (A) It was a retrospective analysis of patients treated in several centers without a central protocol dictating schedule and dose modifications or timing and method of radiological assessments. (B) The clinical sites did not report precise data on different side effects with National Cancer Institute Common Toxicity Criteria scoring, only the date of dose reduction and the reason for it were reported. Nevertheless, we assume that in most cases, the dose was reduced for grade 3 toxicity. (C) Sunitinib plasma level were not available. (D) Correction for multiple testing by Bonferroni, taking into account that the correlation with 11 SNPs was analyzed, indicated a p-value of <0.005 as the threshold for significance. Our results did not reach this level of significance, probably due to the small number of patients in our series. (E) Finally, there was better treatment outcome in our series (PFS 15.0 and OS 29.0 months) compared to the outcome on sunitinib in

the pivotal trial (PFS 11.0 and OS 26.0 months [1]). This difference is likely due to patient selection: all the patients had to complete at least one cycle of sunitinib and had to reach at least the first evaluation by CT scan.

CONCLUSION

Polymorphisms in the *ABCB1* efflux pump are associated with the incidence of dose reductions in mRCC patients treated with sunitinib. Prospective validation of these findings including the association with sunitinib plasma levels is warranted and ongoing (EudraCT: 2011-006085-40/MetaSun).

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TABLE 1A: ANALYZED SNPS LINKED TO SUNITINIB PHARMACOKINETICS

Polymorphism	Number of pts Therapy	Reasons for selection of SNPs for this project
ABCB1 rs1128503 1236C>T rs1045642 3435C>T rs2032582 2677G>T or G>A	88 pts Sunitinib	Better PFS (19 vs 8 months; p=0.027) and OS (34 vs 21 months; p=0.025) in the CC/CT-genotype compared to the TT-genotype in rs1128503 [8].
	89 pts Sunitinib	Trend to better PFS (HR 1.42; p=0.089) and better OS (HR 1.75; p=0.055) in favor of the CC- and CT-genotype in rs1128503 [7].
	129 pts Sunitinib	Better PFS (15.2 vs 8.4 months; p=0.033) and a tendency for prolonged OS (23.9 vs 15.4 months; p=0.078) in presence of a TCG haplotype (rs1045642 – rs1128503 - rs2032582) in ABCB1 (thus a CC-genotype in rs112503) [6].
	241 pts Pazopanib	Better OS (28 vs 20 months, p=0.009) in the CC-genotype compared to the TT-genotype in rs1128503 [11].
CYP3A5 rs776746 6986G>A	128 pts Sunitinib	Better PFS (not reached vs 9.3 months) for the AA- and AG-genotypes compared to the GG-genotypes (p=0.032) [6].
	84 pts Sunitinib	More dose-reductions (HR 3.75; p=0.022) in the AG-genotype compared to the GG-genotype [7].
NR1/2 rs3814055 25385C>T	136 pts Sunitinib	Better PFS (10.8 vs 6.7 months; p=0.025) and better OS (17.1 vs 10.2 months; p=0.017) for the CT- and CC-genotypes compared to the TT-genotype [6].
	241 pts Pazopanib	Better OS for the CC-genotype: 29 vs 22 versus 23 months for the CC-, CT- and TT-variants respectively (p=0.03) [10].
NR1/2 rs2276707 8055C>T	136 pts Sunitinib	Better PFS (10.8 vs 6.7 months) in the CC- and CT-genotypes compared to the TT-genotype (p=0.025) [6].
	83 pts Sunitinib	Better PFS (18 vs 7 months; p=0.047) and trend for better OS (31 vs 12 months; p=0.08) in the GG- and GT-genotype compared to the TT-genotype [8].
NR1/3 rs4073054 7837T>G	135 pts Sunitinib	Better PFS (13.3 vs 8.0 months) if a CAT-copy was absent in the NR1/3 haplotype composed of rs2307424, rs2307418 and rs4073054 (thus no TT-genotype in rs4073054) (p=0.017) [6].
	87 pts Sunitinib	Better PFS (21 vs 12 months; p=0.025) and OS (35 vs 22 months; p=0.035) in the GG- and GT-genotype compared to the TT-genotype [8].

SNP: single nucleotide polymorphism. PFS: progression free survival. OS: overall survival. Pts: patients.

TABLE 1B: ANALYZED SNPS LINKED TO SUNITINIB PHARMACODYNAMICS

Polymorphism	Number of pts Therapy	Reasons for selection of SNPs for this project
VEGFR1 rs9582036 319A>C	91 pts Sunitinib	Better PFS (18 vs 10 months; p=0.06) and better OS (31 vs 14 months; p=0.008) in the AA- and AC-genotypes compared to the CC-genotype [9].
VEGFR3 rs307826 1480A>G	89 pts Sunitinib	Better PFS (13.7 vs 3.6 months; p=0.0079) in the AA-genotype compared to the AG-genotype [7].
	241 pts Pazopanib	OS of 26, 23 and 3.2 months for the AA-, AG- and GG-genotypes, respectively (p=0.04) [11].
	88 pts Sunitinib	Better PFS (19 vs 10 months; p=0.051) and OS (31 vs 22 months; p=0.013) in the AA-genotype compared to the AG- and GG-genotype [8].

SNP: single nucleotide polymorphism. PFS: progression free survival. OS: overall survival. Pts: patients.

TABLE 2: PATIENT CHARACTERISTICS AT DIAGNOSIS OF mRCC AND AT THE START OF SUNITINIB TREATMENT

AT INITIAL DIAGNOSIS		**TOTAL**
Male		72% (69/96)
Mean age		59 years
Ethnic origin	Caucasian	95% (91/96)
	Unknown	5% (5/96)
Synchronous metastasis		51% (47/93)
Fuhrman grade	1-3	46% (43/94)
	4	54% (43/94)
Clear cell histology		92% (88/96)
AT THE START OF SUNITINIB		
ECOG PS >0		42% (40/96)
Neutrophils >4.500/mm^3		43% (40/94)
Platelets >400.000/mm^3		13% (12/96)
Hemoglobin low (<11.5 g/dl (women) or <13 g/dl (men))		43% (41/96)
LDH >1.5 ULN		9% (8/94)
Corrected Calcium >10 mg/dl		8% (7/93)
Time from nephrectomy to systemic treatment <12 months		63% (90/96)
Immunotherapy before sunitinib		25% (24/96)
Site of metastasis	Lung	79% (76/96)
	Liver	20% (19/96)
	Bone	39% (37/96)
	Brain	7% (7/96)
IMDC prognosis	Favorable	14% (13/96)
	Intermediate	59% (57/96)
	Poor	27% (26/96)

ULN: upper limit of normal. ECOG PS: Eastern Cooperative Oncology Group Performance Status. LDH: Lactate deshydrogenase. IMDC: International Metastatic Renal Cell Carcinoma Database Consortium.

TABLE 3: GENOTYPE AND ALLELE DISTRIBUTION OF SELECTED SNPS

RS ID	Polymorphism	Location or functional consequence	n	Wildtype/ Wildtype n (%)	Wildtype/ Variant n (%)	Variant/ Variant n (%)	Observed minor allele frequency (%)	Minor allele frequency in dbSNP (%)
ABCB1								
rs1045642	3435C>T	I1154I	96	27 (28)	49 (51)	20 (21)	46.3	53.4
rs1128503	1236C>T	G412G	95	36 (39)	43 (45)	16 (17)	39.5	45.1
rs2032582	2677G>T or G>A	A893S	89	32 (36)	42 (47)	15 (17)	40.5	41.7
CYP3A5								
rs776746	6986G>A	Affecting splicing	88	78 (89)	11 (8)	0 (0)	6.3	3.6
NR1/2								
rs3814055	25385C>T	UTR-5	93	36 (39)	39 (42)	18 (19)	40.3	33.6
rs2276707	8055C>T	Intron	91	60 (66)	25 (27)	6 (7)	20.3	9.3
NR1/3								
rs2307424	5719C>T	P151P	96	49 (51)	37 (39)	10 (10)	29.6	33.6
rs2307418	7738A>C	Intron	96	71 (74)	25 (26)	1 (1)	14.1	15.9
rs4073054	7837T>G	Intron	96	38 (40)	43 (45)	15 (16)	39.1	40.7
VEGFR1								
rs9582036	319A>C	Intron	96	46 (48)	41 (43)	9 (9)	30.7	31.3
VEGFR3								
rs307826	1480A>G	T494A	96	72 (75)	22 (23)	2 (2)	13.5	10.2

n = number of patients with successful determination of polymorphisms. Note that rs2307424 and rs4073054 in NR1/3 were analyzed because of their involvement in the CAT-haplotype [6]. rs2032582 and rs1045642 in ABCB1 were analyzed because of their involvement in the TCG-haplotype [6].

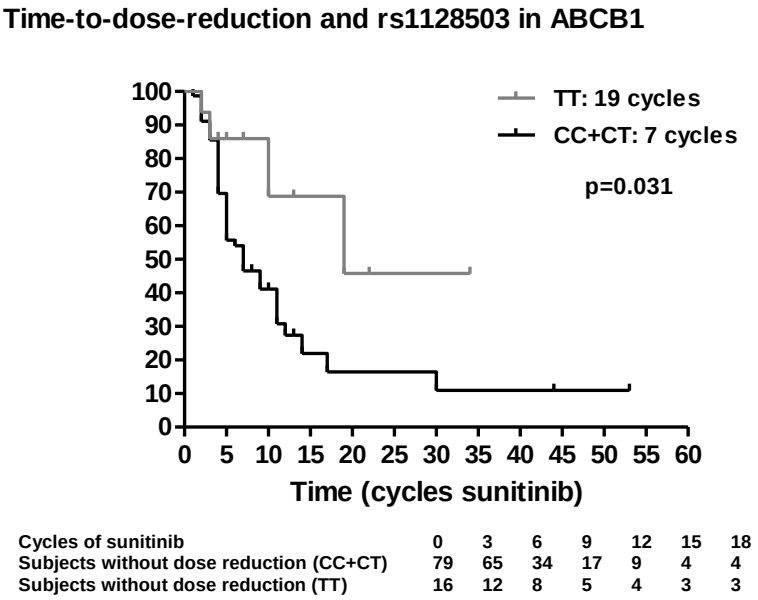
TABLE 4: UNIVARIATE ANALYSIS: ASSOCIATION BETWEEN SNPS AND TIME OF DOSE REDUCTION

Gene (a) SNP ID	Polymor-phism	Nbr of pts	Median TTDR (cycles)	p (UV)	HR	95%CI of HR
ABCB1 rs1128503 1236C>T	CT+CC	73	7	0.031	2.278	1.077 – 4.820
	TT	15	19			
ABCB1 rs1045642 3435C>T	CC	27	12	0.26	NA	NA
	CT	49	7			
	TT	20	19			
	CC+CT	76	9	0.47	NA	NA
	TT	20	19			
ABCB1 rs2032582 2677G>T or G>A	GG	32	11	0.048	NA	NA
	GT/GA	42	5			
	TT/TA	15	19			
	GG+GT/GA	74	7	0.046	2.106	1.015 – 4.371
	TT/TA	15	19			
CYP3A5 rs776746 6986G>A	GG	78	9	0.23	NA	NA
	AG	10	NR			
NR1/2 rs3814055 25385C>T	CC+CT	75	10	0.35	NA	NA
	TT	18	5			
NR1/2 rs2276707 8055C>T	CC+CT	85	7	0.027	2.954	1.132 – 7.707
	TT	6	41.5			
NR1/3 rs2307424 5719C>T	CC	49	11	0.90	NA	NA
	CT+TT	47	7			
NR1/3 rs2307418 7738A>C	AA	71	10	0.92	NA	NA
	AC+CC	25	7			
NR1/3 rs4073054 7837T>G	TT	38	7	0.92	NA	NA
	TG+GG	58	9			
VEGFR1 rs9582036 319A>C	AA+AC	87	9	0.46	NA	NA
	CC	9	5			
VEGFR3 rs307826 1480A>G	AA	72	9	0.85	NA	NA
	AG+GG	24	9			

p-values were calculated by a log-rank test. TTDR: time-to-dose-reduction. UV: univariate analysis. NA: not applicable. HR: hazard ratio. 95%CI: 95% confidence interval.

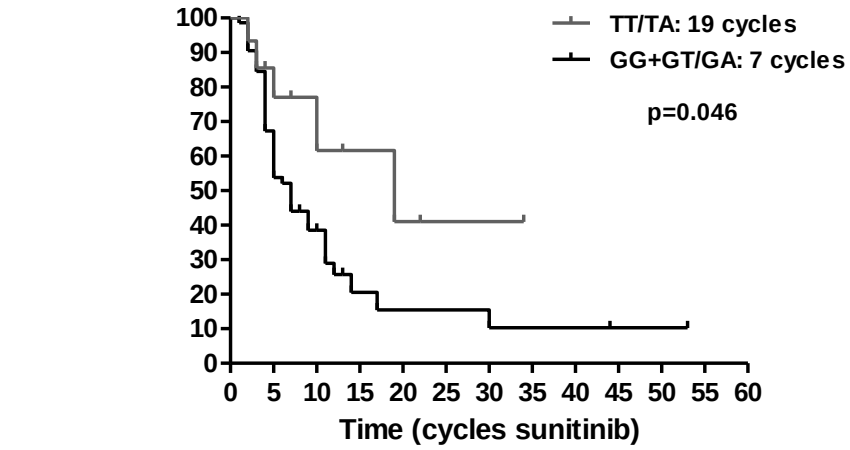
For ABCB1 rs1128503, we analyzed genotype TT against and the combination of genotype CC and CT, because four previous publications clearly associated the TT-variant with poor outcome. For ABCB1 rs1045642 and rs2032582, we analyzed the three genotypes separately and then combined the genotypes in function of the obtained graphs, isolating the groups that were associated to the longest TTDR. In case of CYP3A5, there were no AA-variants in our series. For NR1/2, the variants were combined as it was done in the original publications isolating the patients with TT-genotype, associated with poorer outcome. For NR1/3 rs2307424, the analysis of the three genotypes separately did not result in a significant difference in TTDR. We report the combination of the genotypes as it was done in previous publications. For NR1/3 rs2307418, there was only one patient with the CC-genotype. For NR1/3 rs4073054, we compared the TT-genotype to the TG- and GG-genotype because the TT-genotype was associated to poorer survival. For VEGFR1 rs9582036, the genotypes were pooled as it was done in the original publication. For VEGFR3 rs307826, there were only 2 patients with the GG-genotype: they were pooled with the GA-genotype patients.

FIGURE 1: IMPACT OF ABCB1 rs1128503 VARIANTS ON TIMING OF DOSE REDUCTIONS



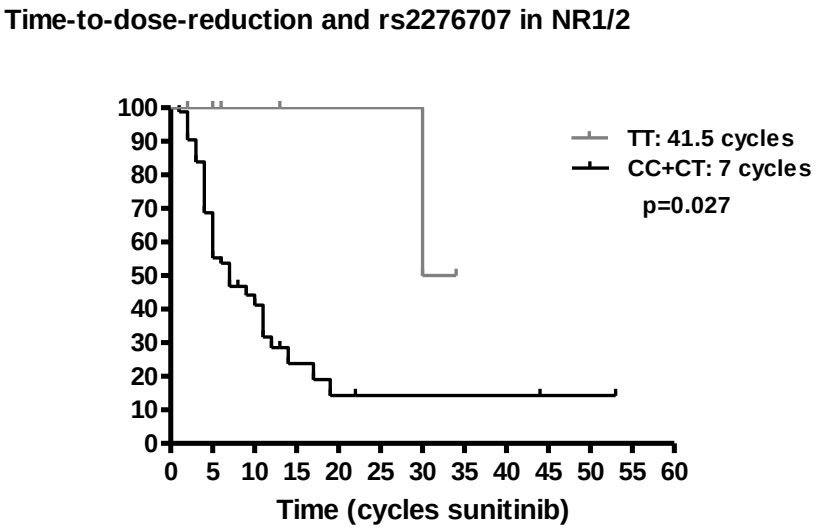
Cycles of sunitinib	0	3	6	9	12	15	18
Subjects without dose reduction (CC+CT)	79	65	34	17	9	4	4
Subjects without dose reduction (TT)	16	12	8	5	4	3	3

FIGURE 2: IMPACT OF ABCB1 rs2032582 VARIANTS ON DOSE REDUCTIONS



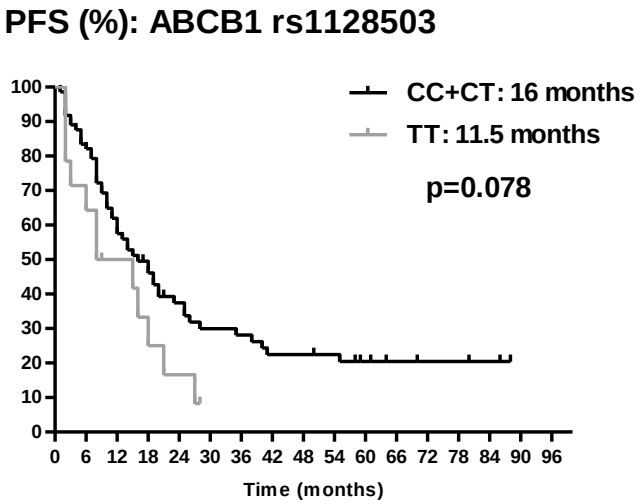
Cycles of sunitinib	0	3	6	9	12	15	18
Subjects without dose reduction (GG+GT/GA)	74	60	31	16	9	4	3
Subjects without dose reduction (TT/TA)	15	12	8	5	4	3	3

FIGURE 3: IMPACT OF NR1/2 rs2776707 VARIANTS ON DOSE REDUCTIONS



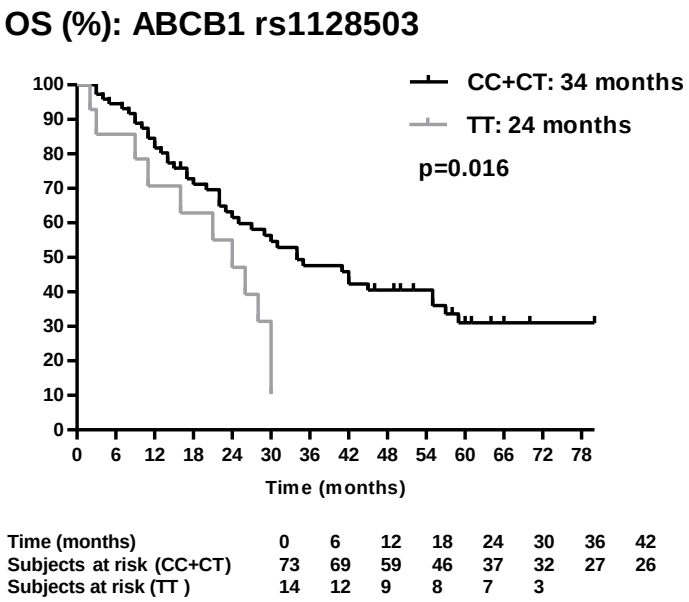
Cycles of sunitinib	0	3	6	9	12	15	18	21
Subjects without dose reduction (CC+CT)	85	68	35	18	10	5	4	3
Subjects without dose reduction (TT)	6	6	4	3	3	2	2	2

FIGURE 4: IMPACT OF ABCB1 rs1128503 VARIANTS ON PROGRESSION FREE SURVIVAL



Time (months)	0	6	12	18	24	30	36
Subjects at risk (CC+CT)	73	60	42	29	22	17	15
Subjects at risk (TT)	14	10	6	4	3		

FIGURE 5: IMPACT OF ABCB1 rs1128503 VARIANTS ON OVERAL SURVIVAL



Time (months)	0	6	12	18	24	30	36	42
Subjects at risk (CC+CT)	73	69	59	46	37	32	27	26
Subjects at risk (TT)	14	12	9	8	7	3		

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PART 5: *VEGFR*1 SINGLE NUCLEOTIDE POLYMORPHISMS ASSOCIATED WITH OUTCOME IN PATIENTS WITH METASTATIC RENAL CELL CARCINOMA TREATED WITH SUNITINIB – A MULTICENTRIC RETROSPECTIVE ANALYSIS

PART 6: PROGNOSTIC IMPACT OF BASELINE SERUM C-REACTIVE PROTEIN IN METASTATIC RENAL CELL CARCINOMA PATIENTS TREATED WITH SUNITINIB.

PART 7: SARCOMATOID DEDIFFERENTIATION IN METASTATIC CLEAR CELL RENAL CELL CARCINOMA AND OUTCOME ON TREATMENT WITH ANTI-VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR TYROSINE KINASE INHIBITORS – A RETROSPECTIVE ANALYSIS

SARCOMATOID DEDIFFERENTIATION IN METASTATIC CLEAR CELL RENAL CELL CARCINOMA AND OUTCOME ON TREATMENT WITH ANTI-VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR TYROSINE KINASE INHIBITORS – A RETROSPECTIVE ANALYSIS

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Keywords: Clear cell renal cell carcinoma, metastases, targeted therapy, outcome, sarcomatoid dedifferentiation

ABSTRACT

Purpose

To assess the efficacy of anti-vascular endothelial growth factor receptor tyrosine kinase inhibitors (anti-*VEGFR*-TKIs) in patients with metastatic clear cell renal cell carcinoma (m-ccRCC) with sarcomatoid dedifferentiation.

Patients and methods

We retrospectively reviewed the files of all m-ccRCC patients consecutively treated with first-line anti-*VEGFR*-TKIs at our institution. Pathology slides from nephrectomy and metastasectomy were assessed for the presence and extent of sarcomatoid dedifferentiation.

Results

One hundred and twenty-four patients were included: nephrectomy and metastasectomy specimens were available in 117 and 35 patients, respectively. Thirty percent of the primary nephrectomy specimens had sarcomatoid features and the median involvement of the sarcomatoid component was 21% (range: 1-95%). Patients with an important sarcomatoid component, defined as $\geq$25% involvement of the tumour, had a very poor outcome: progression-free survival (PFS) and overall survival (OS) were 3 and 6 months, respectively, and no partial responses (PR) were observed. Patients without sarcomatoid dedifferentiation or sarcomatoid involvement <25% had a PFS of 12 months ($p<0.0001$; HR 51, 95%CI 12.58-207.3), an OS of 22 months ($p<0.0001$, HR 10.72, 95%CI 3.56-32.25) and a PR rate of 50% ($p=0.0015$). Patients with a sarcomatoid component $\geq$25% in the metastasectomy also had a poorer PFS and OS on anti-*VEGFR*-TKIs compared to patients with <25% of sarcomatoid features at these sites.

Conclusion

m-ccRCCs-patients with tumours containing a component of sarcomatoid dedifferentiation of $\geq$25% of the total tumour volume have a very poor outcome when treated with anti-*VEGFR*-TKIs. Analysis of the extent of sarcomatoid features in resected metastases can provide additional prognostic information.

INTRODUCTION

At initial diagnosis, up to one-third of renal cell carcinoma (RCC) patients present with metastatic disease and 40% of primary non-metastatic patients, who undergo a nephrectomy with curative intent, will ultimately relapse or develop metastases [1]. Therapies targeting the *VEGF*-pathway (the TKIs sunitinib, sorafenib, pazopanib and axitinib, and the monoclonal antibody bevacizumab) or inhibiting the mammalian target of rapamycin pathway (everolimus and temsirolimus), have almost completely replaced cytokines as first- and second-line treatment of metastatic RCC (mRCC).

In the pivotal trial in previously untreated m-ccRCC patients, half of the patients treated with sunitinib experienced a PR, 43% stable disease (SD), and 7% progressive disease (PD) at first evaluation. Median PFS and OS were 11 and 26.4 months, respectively [2-3]. In the sorafenib pivotal trial in m-ccRCC patients pre-treated with cytokines, 10% of patients achieved a PR, 74% SD and 16% early PD with a median PFS and OS of 5.5 and 17.8 months, respectively [4]. In previously untreated predominantly ccRCC patients, pazopanib showed a median PFS of 11.1 months, and 33% of PR, 42% of SD and 16% of early PD as best response [5]. Despite this major breakthrough in the treatment of mRCC, eventually all patients will relapse due to acquired secondary resistance. Several mechanisms of resistance have been proposed, but reliable biomarkers predictive of anti-*VEGFR*-TKI sensitivity or primary/secondary resistance are still lacking [6]. Baseline serum lactate dehydrogenase (LDH), haemoglobin, corrected calcium, neutrophil, platelet and C-reactive protein (CRP) levels, bone metastases, the number of metastatic sites, prior nephrectomy, Eastern Cooperative Oncology Group Performance Status (ECOG PS) and the interval between diagnosis and systemic therapy were shown to be associated with PFS and/or OS in mRCC treated with anti-*VEGF*-targeted therapy [7-10].

RCCs of all histological subtypes can present with sarcomatoid dedifferentiation, a growth pattern characterized by variable degrees of spindle-shaped cell histology [11-12]. Most sarcomatoid RCCs presents as a bi-phasic lesion with both mesenchymal (sarcoma-like) and epithelial (carcinoma) elements, rarely the sarcomatoid dedifferentiation affects the whole tumour. Sarcomatoid dedifferentiation is associated with a more aggressive disease and poor outcome after nephrectomy or on immunotherapy.

The two major trials that defined the benefit of anti-*VEGFR*-TKIs included patients with clear cell histology [2] or predominantly clear cell histology [5], but did not provide details on the percentage of tumours displaying sarcomatoid elements, nor on the percentage of sarcomatoid involvement of the tumours. In two retrospective series with mRCC-patients with sarcomatoid dedifferentiation, PRs on *VEGF*-targeted therapies were seen, but outcome seemed globally poorer than in mRCC-patients without sarcomatoid dedifferentiation, although a direct comparison was lacking [13-14]. Moreover, both series included also non-ccRCCs, in which anti-*VEGFR*-TKIs are less active [15-16].

We aimed to study the impact of sarcomatoid dedifferentiation on outcome on anti-*VEGFR*-TKIs in clear cell RCC in the metastatic setting.

METHODS

In the database of the University Hospitals Leuven, we searched for patients with m-ccRCC treated with sunitinib, sorafenib or pazopanib as first-line anti-*VEGFR*-TKIs between November 2005 and August 2013 and with available tissue blocks or representative slides from nephrectomy or metastasectomy specimens. Patients in whom only biopsies were available, were not considered for inclusion.

In these patients, anti-*VEGFR*-TKIs were started at the labelled dose: 50 mg/day 4 weeks on 2 weeks off for sunitinib and 800 mg/day continuously for sorafenib and pazopanib. Previous immunotherapy or chemotherapy was allowed, but previous targeted therapy was prohibited. The study was approved by the Ethics Committee of our institution. Signed consent was obtained from all patients. In some cases, we used biologic material and clinical data from patients who had already died and for whom a general positive advice for the utilization of remaining tissue was foreseen by the institutional board.

The following clinical data were assessed: patient age at diagnosis, gender, prior treatment, baseline Karnofsky PS, number and sites of metastases (bone, liver, lung, lymph nodes), platelet count, neutrophil count, LDH level, haemoglobin, corrected calcium, C-reactive protein, and time between nephrectomy and start of systemic treatment. Patients underwent follow up CT-scans (chest and abdomen) every 2 to 3 months during TKI treatment.

All available pathology slides were reviewed by an expert genitourinary pathologist (E.L.) blinded to patient outcome. The classification of RCC subtypes and the presence of sarcomatoid dedifferentiation were assessed following the 2004 WHO Classification of Renal Tumours. The percentage of sarcomatoid elements in each nephrectomy or metastasectomy sample compared to the total available tumour extent was estimated by examining every slide from each case individually. The area of the sarcomatoid component relative to the tumour or metastasis was estimated on each slide. The mean percentage of sarcomatoid component relative to the tumour or metastasis from each slide was added to obtain the total estimated sarcomatoid percentage for each patient. Samples with sarcomatoid dedifferentiation in the original tumour were classified Fuhrman grade 4 by definition, because sarcomatoid dedifferentiation is believed to represent transformation of the RCC to higher grade.

Endpoints of the study were PFS, OS and RR. We defined PFS as the time between the first day on sunitinib and the date of radiological progressive disease or death. Patients who had not progressed at database closure were censored at last follow-up. OS was defined as the time between the first day on sunitinib and the date of death or last date of follow-up. An objective response was defined according to Response Criteria in Solid Tumours (RECIST 1.0). Patients who had PD at the first CT-scan evaluation were considered as early PD patients. Patients who stopped therapy for toxicity before reaching the first evaluation by CT-scan were discarded. PFS and OS distributions were estimated using the Kaplan-Meier product-limit method. p-values were calculated with the log rank Mantel-Cox test. The association between the sarcomatoid component and outcome was studied by a Cox proportional hazards model in order to obtain an optimal cut-off for OS.

RESULTS

One hundred and twenty four patients cases were available for this study including nephrectomy specimens in 117 and metastasectomy specimens in 35 patients. In 28 patients, both nephrectomy and metastasectomy specimens were available. Median follow-up of the 124 patients was 63 months (range 1-96) as calculated from the start of first-line anti-*VEGFR*-TKI.

Analysis of the sarcomatoid component in 117 primary nephrectomy specimens

One hundred and seventeen patients were included in this analysis. Patient characteristics are shown in Table 1. Overall RR was 45% and median PFS and OS were 9 and 18 months, respectively. The mean number of slides reviewed for each patient was 4.95 (range: 1-16). Sarcomatoid dedifferentiation was observed in 35 patients (30%) (Table 2). In tumours with sarcomatoid dedifferentiation, the median percentage of the sarcomatoid component compared to the total available tumour extent was 21% (range: 1%-95%).

When comparing patients with tumours with sarcomatoid dedifferentiation to those without sarcomatoid dedifferentiation, we did not find any statistically significant difference in PFS or OS (HR 1.16 95%CI 0.72-1.88 both for PFS and OS), although there were more early PD patients in the group with sarcomatoid dedifferentiation (34% *versus* 11%, p=0.003 by Fisher exact).

Nevertheless, in a continuous analysis of the impact of the percentage of sarcomatoid dedifferentiation with Cox regression, the presence of a sarcomatoid component was associated with outcome (PFS: HR 22.5 (95%CI 6.8-73.8); p<0.0001 and OS: HR 15.4 (95%CI 4.8-49.8); p<0.0001)) and a threshold of <25% *versus* ≥25% was determined as the best cut-off value for OS. In patients with ≥25% of sarcomatoid dedifferentiation, PFS (3 *versus* 12 months; p<0.0001; HR 51 (95%CI 12.58-207.3)) and OS (6 *versus* 22 months; p<0.0001; HR 10.72 (95%CI 3.56-32.25)) were significantly shorter than in patients with <25% of sarcomatoid dedifferentiation. In patients with a sarcomatoid component ≥25%, we did not observe any PR, while in the group with a sarcomatoid component <25%, we noticed 50 % of PR (p=0.0015). PD was significantly more frequent (64% *versus* 13%; p<0.0001) in patients with a sarcomatoid component ≥25% than in patients with a sarcomatoid component <25%. PRs were frequently observed in patients (16 out of 24 (67%)) with a sarcomatoid component between 1% and 24%. Figure 1 illustrates the impact of the threshold of <25% *versus* ≥25% of sarcomatoid dedifferentiation on PFS and OS.

At start of TKIs, compared to patients with tumours with a sarcomatoid dedifferentiation <25% (Table 4), patients with a sarcomatoid component ≥25% had more often a poor International Metastatic Renal Cell Carcinoma Database Consortium (IMDC) prognostic score, a lower Karnofsky PS, a higher incidence of anaemia, a higher baseline platelets count and a higher baseline CRP plasma level.

Seventy four percent (26/35) of the patients with sarcomatoid dedifferentiation (any percentage of component) started systemic therapy within one year after initial diagnosis of RCC. The median time from diagnosis to systemic treatment in patients with sarcomatoid dedifferentiation (any percentage of component) was 6 months.

Table 3 reports an overview of all established prognostic criteria assessed in our series in univariable analysis and the outcome of the multivariable Cox regression analysis. The presence of a sarcomatoid component ≥25% of the tumour was found to be an independent prognostic factor for PFS and OS with hazard ratios of 4.446 (95%CI 2.084-9.486) and 2.885 (95%CI 1.380-6.028), respectively. Parameters that were taken into account for

the multivariable analysis were: baseline neutrophil and platelets count, baseline haemoglobin, corrected calcium, LDH and CRP levels, Karnofsky PS, and the presence of bone and liver metastases.

Analysis of the sarcomatoid component in 35 metastasectomy specimens

Thirty-five patients were included in this analysis. In all cases, the metastasectomy occurred before the start of anti-*VEGFR*-TKIs. In 14 of these 35 patients (40%), sarcomatoid features were present in the metastasis (Table 2). In four patients with two metastasectomy specimens, the percentage of sarcomatoid dedifferentiation was identical in both metastases. Patients (n=6) with a sarcomatoid component $\geq$25% in the metastasectomy specimen had a very short PFS (2.5 *versus* 23 months; p=0.0007; HR 0.04 (95%CI 0.007-0.26)) and OS (12.5 *versus* 31 months; p=0.0076; HR 0.14 (95%CI 0.036-0.60)) compared to patients (n=29) with a sarcomatoid component <25% (Figure 2).

Comparison of sarcomatoid features in the primary nephrectomy and the metastasectomy specimen

Twenty-eight patients were included in this analysis. Table 4 shows the comparison of the sarcomatoid component in the primary tumour and in the resected metastases. Twenty-six of these 28 patients had a sarcomatoid component of <25% in the primary tumour. Three of these 26 patients had a sarcomatoid component of $\geq$25% in the resected metastases. These three patients had a remarkably poor PFS (3 *versus* 26 months; p=0.0004; HR 0.006 (95%CI 0.00-0.10)) and OS (5 *versus* 31 months; p=0.0009; HR 0.01 (95%CI 0.00-0.16)) on anti-*VEGFR*-TKIs compared to patients with <25% of sarcomatoid features both on the primary nephrectomy specimen and on the resected metastases (Figure 3).

DISCUSSION

The aim of this study was to describe the correlation between the presence of a sarcomatoid component in ccRCC and outcome on anti-*VEGFR*-TKIs in metastatic disease. We identified a subgroup of patients with an important component of sarcomatoid features (>25% of the initial tumor) with very poor outcome when treated with sunitinib, sorafenib or pazopanib. On the opposite, the outcome of patients with tumors with a limited component of sarcomatoid dedifferentiation (<25% of the initial tumor volume) was equivalent to the outcome of patients with tumors without sarcomatoid features.

Our data confirm the association between the presence of a sarcomatoid component and a more aggressive clinical course of the disease. Prior to the era of anti-*VEGF*-targeted therapy, patients with localized sarcomatoid tumours had 2- and 5-year survival rates of only 25%-40% and 19%, respectively [17-18]. In the metastatic setting, median OS was only 3-10 months from the time of diagnosis [19-21]. Some studies reported an association between a higher proportion of sarcomatoid dedifferentiation in the primary kidney tumour and worse survival [18-19] but others did not confirm this observation [20, 22-24]. RCCs with sarcomatoid dedifferentiation tend to metastasize earlier: on 34 nephrectomy specimens, Tickoo et al. reported a significant association between the importance of the sarcomatoid component in the tumour and the incidence of synchronous or metachronous metastases [19]. At the era of anti-*VEGF*-targeted therapy, Golshayan et al. reported a median time from diagnosis to treatment of 7 months in mRCCs with sarcomatoid features. Seventy percent of these patients were diagnosed within 1 year from starting therapy [13].

RCCs with sarcomatoid features have historically demonstrated limited sensitivity to chemotherapy or immunotherapy. Retrospective reviews and a few prospective trials have involved limited patient numbers with disappointing results. On a series of 10 patients with mRCCs with sarcomatoid dedifferentiation treated with doxorubicin and gemcitabine, Nanus et al. reports five patients with early PD, two with SD and three PRs [25]. The largest published prospective clinical trial on 23 sarcomatoid RCCs treated with doxorubicin and ifosfamide reported no objective responses and a median PFS and OS of 2.2 and 3.9 months, respectively [26].

In patients treated with radical nephrectomy for RCC, incidences of sarcomatoid features of 5% to 8% were reported [18, 22, 23]. The higher incidence of sarcomatoid dedifferentiation in our series of primary nephrectomy specimens (30%) is probably explained by the fact that all our patients developed later in the course of the disease or together with the primary tumour distant metastasis requiring systemic therapy. RCCs with sarcomatoid features are more likely to relapse after initial nephrectomy compared to RCCs without sarcomatoid dedifferentiation.

Concerning the relative importance of the sarcomatoid component in each tumour, we found a similar percentage of sarcomatoid dedifferentiation (21% (range 1-95%)) as Golshayan et al. (14% in a series of 43 patients (range 3%-90%)) [13] and Molina et al. (20% in a series of 63 patients (range 2%-100%)) [14]. Both of these series involved patients who were treated in the metastatic setting with systemic therapy.

In literature, two studies indicate that patients with sarcomatoid mRCC (detected on the primary tumour) treated with anti-*VEGF*-targeted therapy can benefit from the therapy, although outcome seems to be poorer than in patients without sarcomatoid dedifferentiation. Golshayan et al. published the largest (n=43) series on outcome in sarcomatoid mRCC treated with sunitinib (49%), sorafenib (28%), bevacizumab (19%) or bevacizumab and sunitinib (5% of patients). PR was observed in 19%, SD in 49% and early PD in 33% of patients. Median PFS and OS were 5.3 and 11.8 months, respectively. PRs were confined to patients with tumours with <20% of sarcomatoid elements. Early PD was observed in only 22% of these patients, while early PD presented in 56% of the patients who had >20% of sarcomatoid elements. The differences in PFS (6.8 *versus* 4.3 months) and OS (14.9 *versus* 8.6 months) favoured the group that had <20% of sarcomatoid elements, but these differences were not statistically significant [13]. Molina et al. reported on 32 patients treated with first-line sunitinib (29 patients) or sorafenib (3 patients). Of the patients treated with sunitinib, 14% had a PR, 59% had SD and 28% PD as best response. Median PFS and OS under sunitinib were 4.4 and 10 months respectively [14]. In opposition to our series, the series Golshayan et al. and Molina et al. did not include a control group of patients without sarcomatoid dedifferentiation. Moreover, patients with non-ccRCCs, in which anti-*VEGFR*-TKIs are less efficient, were included in both series.

We found a similar incidence of sarcomatoid features in metastasectomy specimens as in the primary tumour, but sarcomatoid features were not always stable during the course of the disease. Shuch et al. also compared sarcomatoid aspects in primary nephrectomy and metastasectomy specimens in 32 patients and also reported an evolution of the importance of sarcomatoid features [27]. We are the first to show an association between the importance of the sarcomatoid component in the metastasectomy specimen and outcome on anti-*VEGFR*-TKIs. The study of associations between outcome and characteristics of metastases rather than of the primary tumour is of interest, because in the metastatic setting, response to therapy is usually determined on metastatic sites.

The underlying mechanism of resistance of sarcomatoid tumours to anti-*VEGF*-targeted therapy is unknown, but epithelial-to-mesenchymal transition (EMT) could be involved [28-29]. EMT can occur in several cancer types, including RCCs, and is characterized by the acquisition of a sarcomatoid phenotype with spindle cells and the gradual loss of an epithelial phenotype. Down regulation of micro-RNA-141 was shown to be associated with sarcomatoid features and sunitinib resistance through the induction of EMT and hypoxia resistance [30]. In hepatocellular carcinoma, in vitro experiences have shown that after long-term exposure to sorafenib, resistant cells changed morphologically into spindle shaped cells and showed loss of cell-to-cell contacts, typical features of EMT [31].

Nevertheless, for several reasons anti-*VEGF*-targeted therapy could remain partially active in RCCs with sarcomatoid features. Anti-*VEGF*-targeted therapy can be active on the non-sarcomatoid component of the tumour, inducing tumour shrinkage. EMT is partially reversible and hypoxia-induced pathway markers, as observed by immunohistochemical staining, continue to be over-expressed in sarcomatoid ccRCCs both in the epithelial and in the sarcomatoid component [19].

We noticed a high level of Fuhrman grade 4 tumors in our series: 61% in the total series and 57% in the subgroup with sarcomatoid dedifferentiation <25% of the tumor volume. This high incidence is probably due to the fact that the pathologist looked specifically for sarcomatoid dedifferentiation in the samples. Even tumors with a very limited sarcomatoid component were classified Fuhrman grade 4 by definition.
Our study has two limitations. In patients with few available slides, the percentage of sarcomatoid dedifferentiation might be underestimated. Additionally, given the low number of patients in our study, these findings should be validated in a larger patient series.

CONCLUSION

We studied the impact of sarcomatoid dedifferentiation in primary nephrectomy specimens on treatment outcome in m-ccRCC treated with anti-*VEGFR*-TKIs. We identified a subgroup of patients with an important component of sarcomatoid features ($\geq$25% of the initial tumour) with very poor outcome when treated with sunitinib, sorafenib or pazopanib. Other treatment options should be considered, ideally within clinical trials. Pathologists should not only communicate the presence of sarcomatoid features in RCC resection specimens, but also report the percentage of tumour involvement by the sarcomatoid component. Finally, the presence of sarcomatoid features at metastatic sites can provide additional prognostic information.

SHORT CLINICAL PRACTICE POINTS SECTION

- Metastatic ccRCC-patients with an important component of sarcomatoid features ($\geq$25% of the initial tumour) have a very poor outcome when treated with sunitinib, sorafenib or pazopanib. Other treatment options should be considered, ideally within clinical trials.
- Pathologists should not only communicate the presence of sarcomatoid features in RCC resection specimens, but also report the percentage of tumour involvement by the sarcomatoid component.
- The presence of sarcomatoid features at metastatic sites can provide additional prognostic information.

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CONFLICT OF INTEREST

Benoit Beuselinck received honorarium from Bayer for educational activities. Patrick Schöffski and Pascal Wolter received grants for laboratory, clinical and educational activities from Bayer and GSK. Steven Joniau received honoraria from Bayer and Pfizer. Benoit Beuselinck and Pascal Wolter are investigators of the EudraCT: 2011-006085-40/MetaSun trial supported by Pfizer.

TABLE 1: INCLUDED PATIENTS WITH AVAILABLE PRIMARY NEPHRECTOMY SPECIMEN (117)

PATIENT CHARACTERISTICS AT INITIAL DIAGNOSIS		TOTAL	Sarcomatoid component <25%	Sarcomatoid component ≥25%	p (*)
Number of patients		117	91% (106/117)	9% (11/117)	
Mean age (years)		59	59	59	
Male		66% (77/117)	65% (69/106)	73% (8/11)	0.6
Metastases at initial diagnosis		50% (58/117)	47% (50/106)	73% (8/11)	0.1
Fuhrman (**)	Grade 1-3	39% (46/117)	43% (46/106)	0% (0/11)	0.005
	Grade 4	61% (71/117)	57% (60/106)	100% (11/11)	
PATIENT CHARACTERISTICS AT START OF TKI					
Karnofsky <80		14% (16/117)	11% (12/106)	36% (4/11)	0.02
Neutrophils >4.500/mm³		51% (60/117)	50% (53/106)	64% (7/11)	0.4
Platelets >400.000/mm³		14% (16/117)	11% (12/106)	36% (4/11)	0.02
Hemoglobin <11.5 g/dl (women) or <13 g/dl (men)		47% (55/117)	42% (45/106)	91% (10/11)	0.003
LDH > 1.5x ULN		5% (6/117)	6% (6/106)	0% (0/11)	0.4
Corrected calcium > 10 mg/dl		25% (29/117)	23% (24/106)	45% (5/11)	0.1
CRP ≥ 5 mg/l		75% (88/117)	73% (77/106)	100% (11/11)	0.046
Time nephrectomy to systemic treatment <12 months		50% (58/117)	49% (52/106)	55% (6/11)	0.7
Immunotherapy before TKI		38% (45/117)	39% (41/106)	36% (4/11)	0.9
Site of metastasis	Lung	77% (90/117)	76% (81/106)	82% (9/11)	0.7
	Liver	21% (24/117)	20% (21/106)	27% (3/11)	0.6
	Brain	7% (8/117)	6% (6/106)	18% (2/11)	0.1
	Bone	42% (49/117)	42% (44/106)	45% (5/11)	0.8
	Mean number	2.93	2.91	3.18	
IMDC prognosis	Favourable	11% (13/117)	12% (13/106)	0% (0/11)	0.2
	Intermediate	51% (60/117)	55% (58/106)	18% (2/11)	0.02
	Poor	38% (44/117)	33% (35/106)	82% (9/11)	0.002
FIRST-LINE TKI					
Sunitinib		71% (83/117)	70% (74/106)	82% (9/11)	0.4
Sorafenib		18% (21/117)	20% (21/106)	0% (0/11)	0.1
Pazopanib		11% (13/117)	10% (11/106)	18% (2/11)	0.4

NA: Not applicable. ULN : Upper limit of normal. TKI : Tyrosine kinase inhibitor. IMDC : International metastatic renal cell carcinoma database consortium.
(*) P-values were obtained by Fisher Exact test for comparison of percentages.
(**) All samples with sarcomatoid dedifferentiation were classified Fuhrman grade 4.

TABLE 2: INCIDENCE OF SARCOMATOID DEDIFFERENTIATION

SARCOMATOID INVOLVEMENT	n (%) PRIMARY TUMOUR	n (%) METASTASECTOMY	p-value
No sarcomatoid dedifferentiation	82/117 (70%)	21/35 (60%)	0.26
Sarcomatoid dedifferentiation	35/117 (30%)	14/35 (40%)	
1-10%	23/117 (20%)	6/35 (17%)	
11-24%	1/117 (1%)	2/35 (6%)	
25-50%	5/117 (4%)	4/35 (11%)	
>50-100%	6/117 (5%)	2/35 (6%)	
≥25%	11/117 (9%)	6/35 (17%)	0.15

TABLE 3: UNIVARIATE AND MULTIVARIATE ANALYSIS FOR PREVIOUSLY DESCRIBED PROGNOSTIC FACTORS IN THE SERIES OF 117 NEPHRECTOMIES

UNIVARIATE ANALYSIS		PFS (months)	p-value (*)	OS (months)	p-value (*)
Sarcomatoid dedifferentiation	<25% (n=106)	12	<0.0001	22	<0.0001
	≥25% (n=11)	3		6	
	Continuous analysis		<0.0001 HR 22.5 (6.8-73.8)		<0.0001 HR 15.4 (4.8-49.8)
Baseline neutrophils	≤4.500/mm³ (n=57)	14	0.02	22	0.03
	>4.500/mm³ (n=60)	6		14	
Baseline serum calcium	≤10 mg/dl (n=88)	9	0.6	20	0.19
	>10 mg/dl (n=29)	9.5		11	
Baseline hemoglobin	≥11.5 g/dl in women / ≥13.0 g/dl in men (n=62)	15	0.0005	29	<0.0001
	<11.5 g/dl in women / <13.0 g/dl in men (n=55)	5		9	
Baseline platelet count	≤400.000 (n=101)	11	0.008	20	0.01
	>400.000 (n=16)	3		7	
Baseline lactate dehydrogenase	≤1.5x ULN (n=111)	11	0.009	20	0.01
	>1.5x ULN (n=6)	2		4	
Baseline CRP	<5 mg/l (n=29)	27	<0.0001	31	<0.0001
	>5 mg/l (n=88)	6		12	
Interval diagnosis to systemic treatment	<12 months (n=58)	8	0.7	20	0.9
	>12 months (n=59)	9		18	
Baseline Karnofsky PS	≥80 (n=101)	11	0.004	22	0.0009
	<80 (n=16)	3		5	
Presence of bone metastases	No (n=68)	10	0.4	20	0.07
	Yes (n=49)	9		18	
Presence of liver metastases	No (n=93)	11	0.008	22	0.008
	Yes (n=24)	3		6.5	
IDMC prognostic score	Favorable (n=13)	17	0.0006	35	<0.0001
	Intermediate (n=60)	14		26	
	Poor (n=46)	4		6.5	
TKI	Sunitinib (n=83)	12	0.4	18	0.8
	Sorafenib (n=21)	5		12	
	Pazopanib (n=13)	7		10	

MULTIVARIATE ANALYSIS FOR PFS	p-value	Hazard Ratio	95%CI	
Sarcomatoid dedifferentiation 0-<25% *versus* ≥25%	<0.0001	4.446	2.084	9.486
Baseline CRP ≤5mg/l *versus* >5mg/l	0.006	2.321	1.268	4.249
MULTIVARIATE ANALYSIS FOR OS				
Sarcomatoid dedifferentiation 0-<25% *versus* ≥25%	0.005	2.885	1.380	6.028
Baseline CRP ≤5mg/l *versus* >5mg/l	0.011	2.263	1.202	4.262

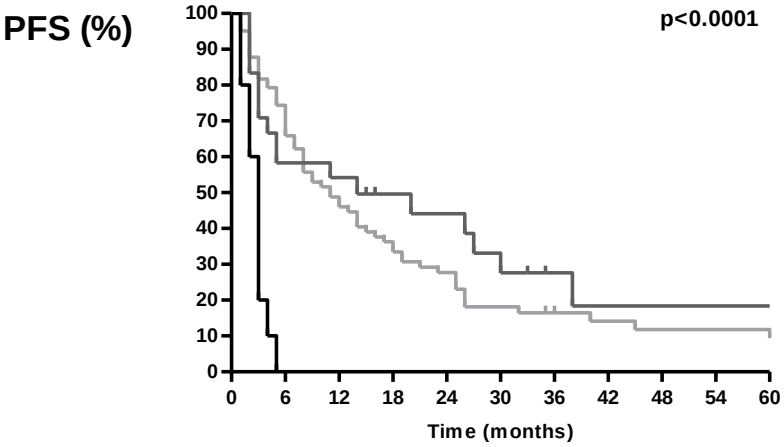
ULN: upper limit of normal. CRP: C-reactive protein. Karnofsky PS: Karnofsky performance status. IMDC : International metastatic renal cell carcinoma database consortium. TKI: tyrosine kinase inhibitor. PFS: progression-free survival. OS: overall survival. HR: Hazard Ratio. 95%CI: 95% Confidence Interval. (*) Log-rank comparison between Kaplan-Meier curves.

TABLE 4: COMPARISON OF THE SARCOMATOID COMPONENT BETWEEN THE PRIMARY KIDNEY TUMOUR AND THE RESECTED METASTASES

Patient	Sarcomatoid component on nephrectomy	Sarcomatoid component on metastasectomy	Organ of metastasectomy	Evolution of the sarcomatoid component
1+2+3	0%	0%	Bone	Stable low
4+5	0%	0%	Skin	Stable low
6	0%	0%	Lymph node	Stable low
7	0%	0%	Gallbladder	Stable low
8+9	0%	0%	Lung	Stable low
10+11	0%	0%	Pancreas	Stable low
12+13	0%	0% 0%	Adrenal Lung	Stable low
14	0%	0% 0%	Lung Thyroid	Stable low
15	0%	1%	Skin	Stable low
16	0%	1%	Liver	Stable low
17	0%	1%	Bone	Stable low
18	1%	0%	Lymph node	Stable low
19	1%	0% 0%	Bone Adrenal	Stable low
20	0%	5%	Lung	Stable low
21	5%	0%	Duodenum	Stable low
22	10%	5%	Local relapse	Stable low
23	0%	20%	Parietal	Stable low
24	0%	30%	Gingiva	Up
25	0%	100%	Liver	Up
26	0%	50%	Bone	Up
27	25%	90%	Gingiva	Stable high
28	60%	0%	Lung	Down

NOTE: “Stable low” indicates that the sarcomatoid component is <25% in both the primary nephrectomy as in the metastasectomy specimen. “Stable high” indicates that in both samples, the sarcomatoid component is ≥25%. “Up” means that the sarcomatoid component was <25% in the primary nephrectomy specimen and ≥25% in the metastasectomy. “Down” indicates that the sarcomatoid component was ≥25% in the primary nephrectomy specimen and <25% in the metastasectomy.

FIGURE 1: Impact of sarcomatoid dedifferentiation in 117 primary nephrectomy specimens on progression-free-survival and overall survival

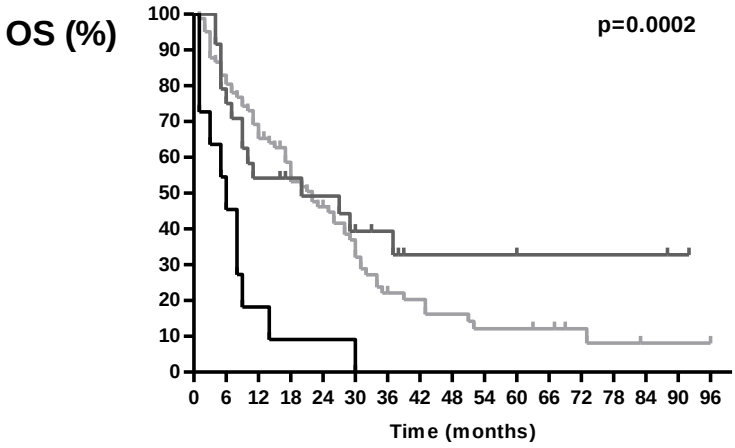


Months	0	6	12	18	24	30	36	42
No sarcomatoid component	82	61	35	26	18	11	9	6
Sarcomatoid component 1-<25%	24	14	12	9	8	6	3	2
Sarcomatoid component ≥25%	11	0	0	0	0	0	0	0

0% Sarcomatoid component: mPFS 11 months

1-<25% Sarcomatoid component: mPFS 14 months

≥25% Sarcomatoid component: mPFS 3 months



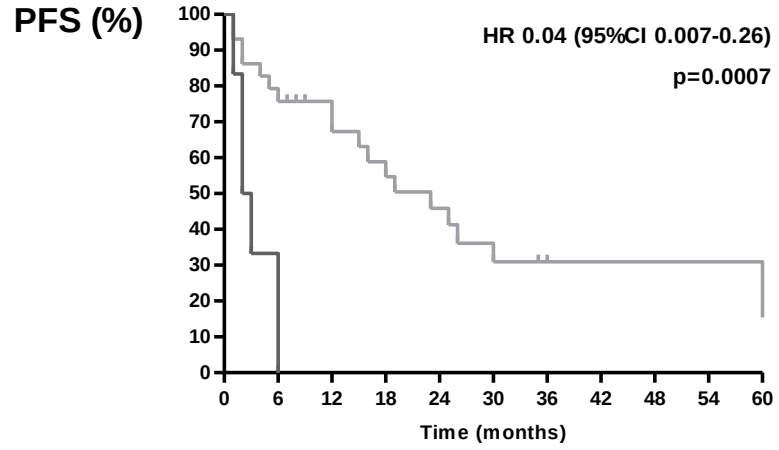
Months	0	6	12	18	24	30	36	42	48	54	60	66
No sarcomatoid component	82	68	54	43	32	23	13	10	8	6	6	5
Sarcomatoid component 1-<25%	24	19	13	11	10	8	6	3	3	3	3	2
Sarcomatoid component ≥25%	11	6	2	0	1	1	0	0	0	0	0	0

0% Sarcomatoid component: mOS 22 months

1-<25% Sarcomatoid component: mOS 20 months

≥25% Sarcomatoid component: mOS 6 months

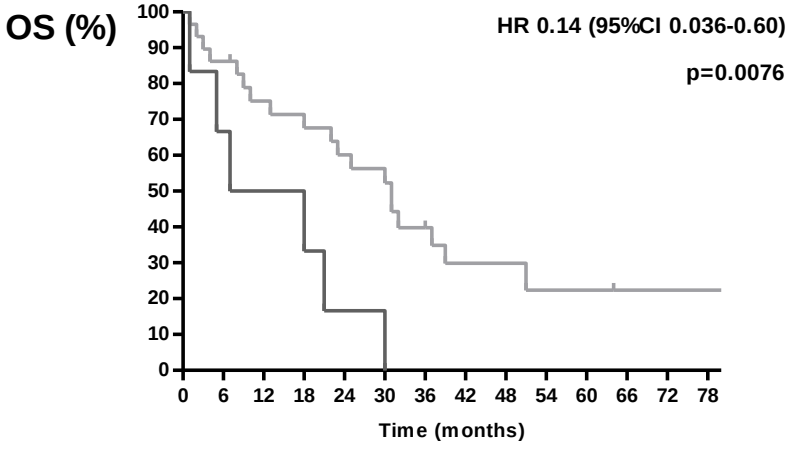
FIGURE 2: Impact of sarcomatoid dedifferentiation in 35 metastasectomy specimens on progression-free-survival and overall survival



Months	0	6	12	18	24	30	36
Sarcomatoid component 0-<25%	29	22	18	14	10	7	4
Sarcomatoid component ≥25%	6	1	0	0	0	0	0

Sarcomatoid component 0-<25%: mPFS 23 months

Sarcomatoid component ≥25%: mPFS 2.5 months

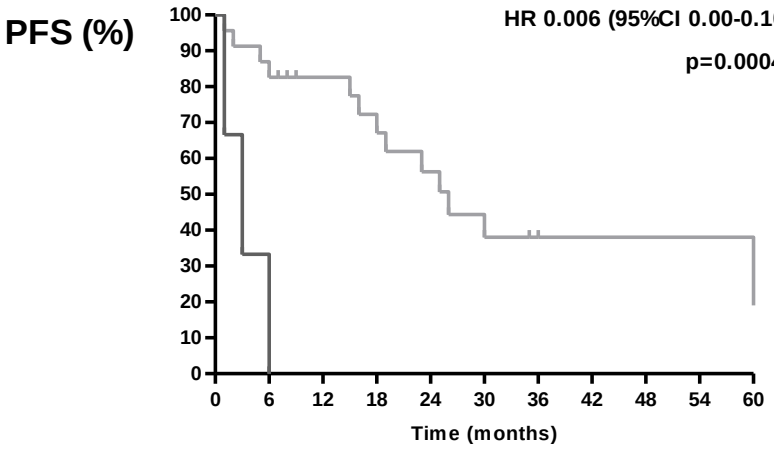


Months	0	6	12	18	24	30	36	42	48	54
Sarcomatoid component 0-<25%	29	25	20	19	16	14	9	4	4	3
Sarcomatoid component ≥25%	6	4	3	3	1	1	0	0	0	0

Sarcomatoid component 0-<25% mOS 31 months

Sarcomatoid component ≥25% mOS 12.5 months

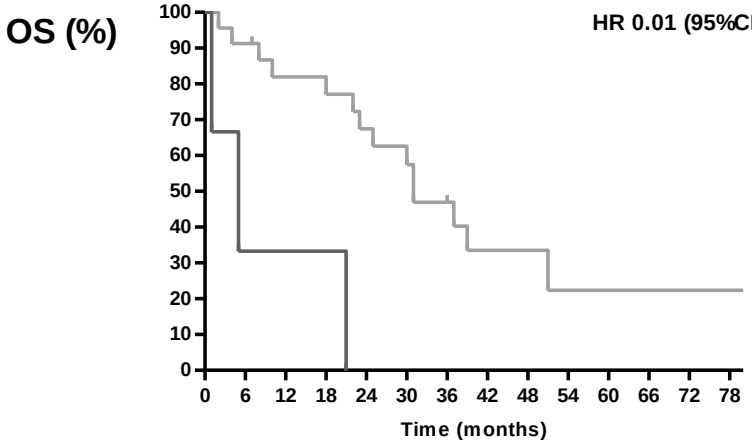
FIGURE 3: Impact of the importance of sarcomatoid dedifferentiation (0-<25% *versus* ≥25%) in the metastasectomy specimen (M) in 26 patients without or with limited sarcomatoid features (0-<25%) in the primary tumour (T)



Months	0	6	12	18	24	30	36
Sarcomatoid component 0-<25% on T and M	23	20	16	14	10	7	4
Sarcomatoid component 0-<25% on T but ≥25% on M	3	1	0	0	0	0	0

Sarcomatoid component 0-<25% on T and on M: mPFS 26 months

Sarcomatoid component 0-<25% on T but ≥25% on M: mPFS 3 months



Months	0	6	12	18	24	30	36	42	48
Sarcomatoid component 0-<25% on T and on M	23	21	17	17	14	12	8	3	3
Sarcomatoid component 0-<25% on T but ≥25% on M	3	1	1	1	0	0	0	0	0

Sarcomatoid component 0-<25% on T and on M: mOS 31 months

Sarcomatoid component 0-<25% on T but ≥25% on M: mOS 5 months

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PART 8: MOLECULAR SUBTYPES OF CLEAR CELL RENAL CELL CARCINOMA ARE PREDICTIVE OF RESPONSE TO SUNITINIB IN THE METASTATIC SETTING

MOLECULAR SUBTYPES OF CLEAR CELL RENAL CELL CARCINOMA ARE PREDICTIVE OF RESPONSE TO SUNITINIB IN THE METASTATIC SETTING

We are currently working on the definitive manuscript of the results of this part of our work. Therefore, in this thesis manuscript, we only give a summary of our most important findings.

The objective of this part of our project was to identify new predictive molecular markers for outcome in m-ccRCC patients treated with sunitinib, through a multi-omics analysis (mRNA-expression, genome and methylome) combined with clinical and pathological data as well as an analysis of the two most frequent mutations in ccRCC: *VHL* and *PBRM1*.

121 ccRCCs were collected from patients surgically treated in 19 French and one Belgian surgical departments from 1994 to 2011. All these patients developed synchronous or metachronic metastases and were treated in the metastatic setting with sunitinib as first-line anti-*VEGF*-targeted therapy. Global mPFS was 13 months and global mOS 27 months.

Based on mRNA expression data, we identified 4 robust molecular subgroups of ccRCCs: ccrcc1, -2, -3 and -4, with a relative frequency of 33%, 41%, 11% and 15%, respectively. This molecular transcriptomic classification of m-ccRCC is in agreement with the previously published transcriptomic analyses of Brannon et al. [1][2], who identified three subgroups: ccA, ccB and Cluster_3, with a refinement of ccB in two sub-entities in our classification. Our subgroup ccrcc1 and 4 correspond to Brannon's ccB, our ccrcc3-subgroup to Cluster_3 and our-ccrcc2 subgroup to Brannon ccA. In order to identify these molecular subgroups we developed a 35-gene signature that could be useful to screen ccRCCs in the next future.

mPFS and mOS were significantly different among the four subtypes (p=0.001 and 0.0003, respectively). The ccrcc3-tumours displayed the best mPFS (24 months) followed by ccrcc2-tumours (19 months), ccrcc1-tumours (13 months) and ccrcc4-tumours (8 months). For mOS, the corresponding data are: 50 months for ccrcc3-tumours, 35 months for ccrcc2-tumours, 24 months for ccrcc1-tumours and 14 months for ccrcc4-tumours. The classification was the most significant covariate in univariate cox analysis and remained the only significant covariate in the multivariate cox model.

Response to sunitinib defined by RECIST was clearly associated to the ccrcc-classification: ccrcc3-patients had a RR of 70%. In ccrcc2-, ccrcc1- and ccrcc4-patients this was respectively 53%, 41% and 20%. 27% of the ccrcc4-patients and 23% of the ccrcc1-patients were progressive at the first CT-scan evaluation, compared to 3% for ccrcc2-patients. All ccrcc3-patients had clinical benefit. All the complete and long lasting PR (>44 months of PFS) patients were found in the ccrcc2- and ccrcc3-subgroups.

Although we do not have a placebo-treated control group, the important and significant difference in RECIST response rate among subgroups admits us to state that these molecular subtypes have a predictive value for response to sunitinib.

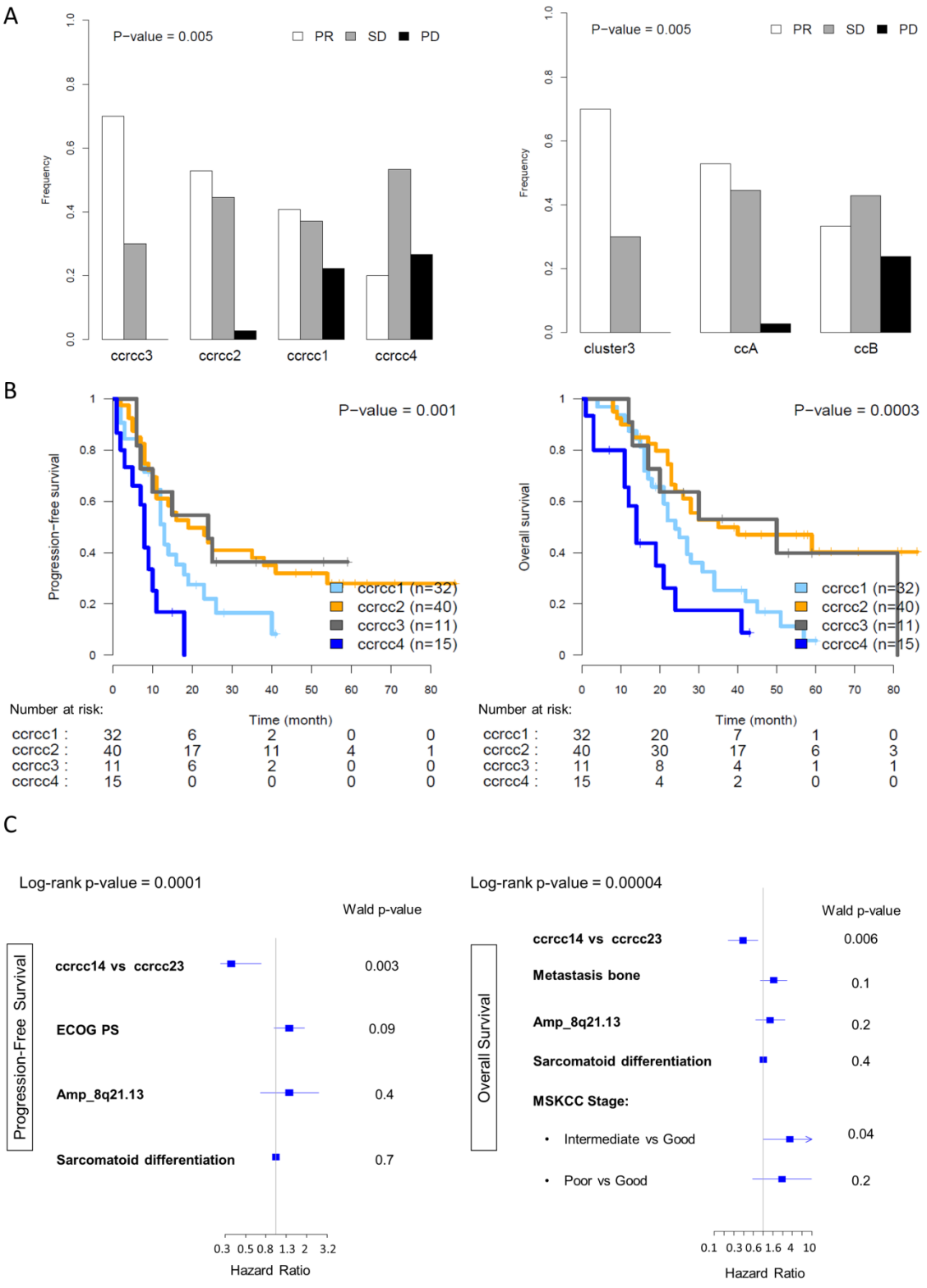

Figure 2: (A) Association of sunitinib response with the unsupervised subgroups ccrcc1-ccrcc4 (left) and the Brannon subgroups (right). The p-values result from Fisher-exact tests. (B) Association of the four unsupervised subgroups with progression-free survival (PFS) (left) and overall survival (OS) (right). Log-rank p-values are on the top right. (C) Forest plots of the multivariate cox models for PFS and OS. For each pair of correlated covariates, a bivariate cox model was first built to select the more significant covariate to integrate in the multivariate cox model.

The molecular characterization of these subgroups leaded to the identification of specific characteristics in each subgroup, some of them associated directly with outcome.

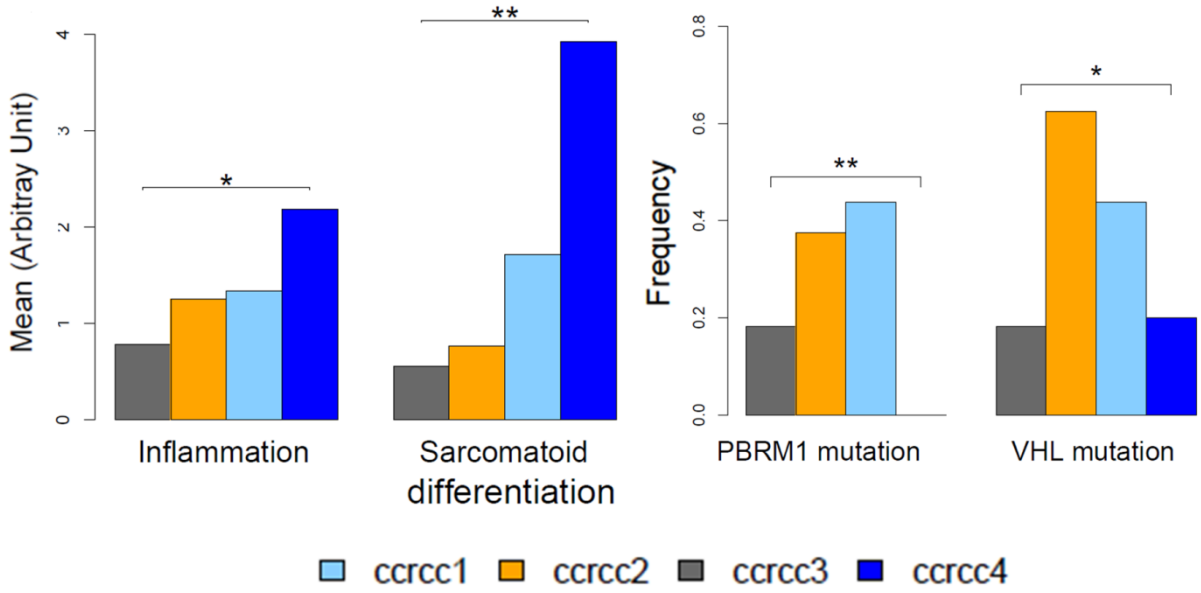

Figure 3: Barplot of the pathologic-molecular covariates associated with the four unsupervised subgroups (ccrcc1 to ccrcc4). Stars correspond to significant anova or fisher p-values: *** p-value<0.001, ** p-value<0.01 and * p-value<0.05.

The two subtypes enriched in non-responders to sunitinib, ccrcc1 and ccrcc4, shared common molecular characteristics such as up-regulation of MYC-targets or a hyper-methylated status strongly correlated with a polycomb stem cell phenotype. The ccrcc4-subtype showed specific pathological features such as more inflammation and sarcomatoid phenotypes. These findings are in accordance with our findings described in chapter 6 and 7, respectively. It also showed an up-regulation of cellular immune pathways and an omnipresent 8q21.13 amplification. Tumours classified in ccrcc4 and ccrcc1 were less frequently mutated at *PBRM1* and *VHL* genes. ccrcc2-tumours were most often *VHL*-mutated.

Among the groups of tumours enriched in responders to sunitinib, the expression profile of ccrcc3-samples was similar to that of normal kidney samples concerning metabolic pathways and transporter activities, similarly to the Cluster_3 described by Brannon [2]. The ccrcc3-subgroup also showed a methylation profile similar to that of normal kidney samples. Hypoxia pathways were not activated in ccrcc3-tumours. The ccrcc2-subgroup was not characterized by specific pathways; it always showed an intermediate expression signature, comprised between ccrcc3 and (ccrcc1+ccrcc4) related profiles. In ccrcc2-tumours cellular response to hypoxia was not as important as in the ccrcc1- and ccrcc4-subtypes.

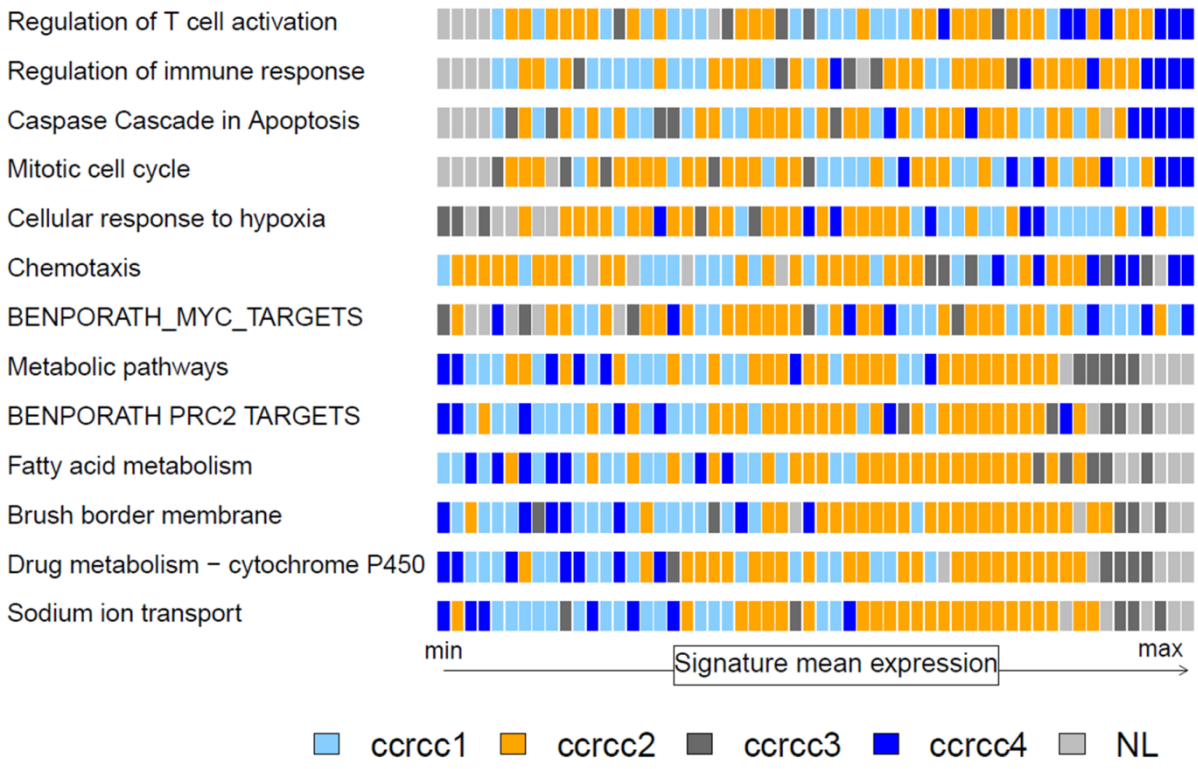

Figure 4: Representation of the mean expression level of differentially regulated pathways between the four subgroups. Pathways are sorted by the difference between the ccrcc4-subgroup and the normal sample (NL). For a given pathway, samples are sorted by mean expression value.

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CONCLUDING DISCUSSION AND PERSPECTIVES

Several years after approval of anti-VEGF-targeted therapy for locally advanced and metastatic ccRCC, investigation is running in order to predict responses on these therapies and in order to determine the reasons of primary or secondary resistance. The aim of this study was to discover new clinical, biochemical, pathological, molecular and genetic markers predictive for response and/or prognostic in m-ccRCC patients treated with anti-VEGFR-TKIs.

1. The contribution of our work to this research field

This research project contributes to a better understanding of the mechanisms of sensitivity or resistance to anti-VEGFR-TKIs in m-ccRCC and to a more precise estimation of the RR, PFS and OS in m-ccRCC patients treated with anti-VEGFR-TKIs.

1. We were among the first to describe the negative impact of the presence of bone metastasis in m-ccRCC on outcome. The presence of bone metastasis does not seem to have an important predictive value, but rather a prognostic value. We also observed that patients with metastatic spread limited to the lungs and lymph nodes had the best outcome and that the combination of bone and liver metastases correlated with poor outcome. Since the publication of our results in 2010, several other publications reported on the negative impact of bone metastases on outcome. In a pooled analysis of 1.067 predominantly clear cell mRCCs treated with sunitinib in six trials, the presence of bone metastases was significantly associated with lower RR and poorer OS (148). In a retrospective analysis of 2.027 mRCC patients treated with targeted therapies, the presence of bone and/or liver metastases correlated with poorer outcome. Patients combining bone and liver metastases had a particularly poor outcome (149). Possible mechanisms that could explain why the presence of bone metastases are an adverse factor for outcome are: (A) the vicious circle of mutual stimulation between osteoclasts and tumour cells in the bone. (B) RCCs with bone metastases could be a more aggressive subtype of RCCs. (C) Anti-VEGFR-TKIs could be less efficient in the bone micro-environment than at other metastatic sites and (D) in case of bone metastasis, the treatment with anti-VEGFR-TKIs could be more often interrupted due to skeletal related events. In accordance with mechanism (A), we could describe the positive impact of concomitant therapy associating anti-VEGFR-TKIs with bisphosphonates, which are bone resorption inhibitors in presence of bone metastases.

2. We added evidence that polymorphisms in genes involved in sunitinib pharmacokinetics and more precisely in the ABCB1 efflux pump, have an impact on outcome in m-ccRCCs treated with sunitinib. Polymorphisms in the efflux pump are thought to influence sunitinib plasma levels. Although we did not have access to dosing of sunitinib plasma levels, we have indirect evidence for such a relationship. Patients with the variant associated with a more active efflux pump, had not only a poorer outcome on sunitinib, but also a longer time-to-dose-reduction, indicating that in these patients the sunitinib plasma levels might be lower than in patients with the wild-type *ABCB1*. If these findings would be validated in future trials, they could lead to the identification of a subgroup of patients in which a dose escalation to 62.5 mg/day and even 75 mg/day of sunitinib could safely be performed. We also validated previously published associations between polymorphisms in the cytochrome

CYP450 3A4 regulators NR1/2 and *NR1*/3 and outcome on sunitinib. As these polymorphisms are associated with TKI-kinetics and not with the malignancy of the tumour, they are not prognostic, but they have a predictive impact.

3. We validated the impact of polymorphisms in *VEGFR3* on outcome in m-ccRCCs treated with sunitinib and we were the first team to show the association between polymorphisms in *VEGFR1* and outcome. Patients with the SNP rs307826 variant in *VEGFR3* have a poorer outcome when treated with sunitinib. This finding is of interest because in the meantime, it was shown by Garcia-Donas et al. that patients with the variant *VEGFR3* rs307826 polymorphism had a lower expression of VEGFR3 (145). Moreover, VEGFR3 is highly expressed in tip cells, which are necessary for the sprouting of new vessels (150). Our findings concerning the polymorphism rs9582036 in *VEGFR1* are also interesting, because in pancreatic adenocarcinomas treated with chemotherapy with or without bevacizumab, this variant was shown to be a predictive factor.

4. We published the largest series in literature analysing the impact of baseline serum C-reactive protein (CRP) levels on outcome in m-ccRCC patients treated with sunitinib. Baseline CRP-levels were associated with PFS and OS on multivariate analysis. We were able to improve the accuracy of the IMDC prognostic score by the addition of baseline CRP-levels. As most of the clinical and biological parameters associated with outcome, we think that this biomarker has a prognostic value and that high CRP-levels are associated with a more aggressive disease. Accordingly, on the contrary, we found a RR as high as 72% in patients with a good IMDC prognostic score and normal baseline CRP-levels. Thus, this parameter might also have an additional predictive value.

5. We added evidence that sarcomatoid dedifferentiation in 25% or more of the tumoral volume of the primary tumour has a negative impact on outcome in m-ccRCC patients treated with anti-VEGFR-TKIs. Moreover, our team recently published that in a series of 20 ccRCC patients treated with sunitinib, microRNA-141 was significantly down-regulated in tumours poorly responding to sunitinib. This seemed spatially linked to EMT in vivo. Reintroduction of microRNA-141 in vitro reversed EMT and decreased cell viability in hypoxic conditions (151).

6. Finally, through our multi-omics analysis of 121 ccRCC tumours, we were capable for the first time to show the predictive value of a mRNA expression profile based classification of ccRCCs on outcome when treated with sunitinib in the metastatic setting. Our classification is similar to formerly published classifications showing an impact on prognosis after nephrectomy. Nevertheless, we described significant differences in RR among the subgroups, showing that on top of a prognostic value, this classification has also a predictive value. In our analysis, tumours without activation of pathways involved in cellular response to hypoxia or with a limited activation of these pathways responded better than tumours with an important activation of these pathways. On the opposite, upregulation of genes associated with immune response as well as the importance of immune infiltrates in the tumour were associated with poorer outcome, a finding that can easily be correlated with the association between baseline CRP-levels and outcome. The subgroup with the poorest outcome had an important incidence of sarcomatoid dedifferentiation, chromosome 8q amplifications and c-myc target transcription and a stem cell phenotype.

Unfortunately, up to now, not any single parameter allows us to predict efficacy or resistance to anti-VEGFR-TKIs nor to preclude some patients from therapy with anti-VEGFR-TKIs. Efficacy or resistance seem rather to be the

result of multiple parameters. In the absence of a single predictive biomarker, these parameters could be combined in scoring systems predicting the probability of sensitivity (response) or resistance.

2. New hypothesis on the working mechanisms of anti-VEGFR-TKIs and of resistance

Taking together different experimental observations, we can emit the following hypotheses on the mechanism of action of anti-VEGFR-TKIs and on mechanisms of resistance.

2.1. Inhibition of the activated VEGF-pro-angiogenic pathway in hyper-vascular tumours

The efficacy of anti-VEGF-targeted therapy in m-ccRCC is in part explained by the inhibition of the activated VEGF-pro-angiogenic pathway in these hyper-vascular tumours. The arguments are the following:

- ccRCCs are the tumours that most express VEGF, through omnipresent VHL-dysfunction. Therefore, neo-angiogenesis is particularly important in ccRCC, which are hyper-vascular tumours. Moreover, anti-VEGF-targeted therapy is clinically less useful in non-ccRCCs, which are tumours without VHL-impairment (72, 74).
- Anti-VEGFR-TKIS with high affinity for VEGFR1, -2 and -3 such as axitinib as well as purely anti-VEGF-directed antibodies such as bevacizumab have proven efficacy in m-ccRCC.
- After the start of anti-VEGFR-TKIs, normalization of anarchic neo-vessels occur.
- Several reports suggest an association between the levels of expression of proteins of the VEGF-pathway and outcome.

2.2. Induction of normoxia, rather than increase of hypoxia

There are arguments to believe that efficacy of anti-VEGF-targeted therapy is probably associated with induction of normoxia, rather than with increase of hypoxia, through a regression, but most of all normalization of the tumoral vasculature, with lowering of the interstitial pressure and better oxygen delivery. The arguments are the following:

- Tumour hypoxia is known to be a factor of poor prognosis because it renders a tumour more aggressive, leading to higher levels of HIF and thus higher expression of genes involved in cell cycle, invasion, and progression in tumour vasculature, tumour proliferation, and metastatic spread. Thus, hypoxia should be avoided and normoxia should be the target of therapy. Through the reduction and normalization of blood vessels, anti-VEGF-targeted therapy seems to lead to better oxygen delivery in the tumour, because the newly formed vasculature is poorly functional, with tortuous immature vessels, anarchic interconnections, increased capillary permeability, and fluid leakage into the interstitial space resulting in increased interstitial pressure, impairing oxygen diffusion and thus inducing tumour hypoxia. As a consequence, restoring normoxia or at least lowering hypoxia seems to be an important part of the mechanism of

action of anti-VEGF-targeted therapy. Moreover, tumours that remain hypoxic under VEGF-blockade, will activate VEGF-independent neo-angiogenesis. In normoxic tumours there will be no need for the activation of alternative pro-angiogenic pathways.

- Recent work by Hugonnet et al. seems to confirm this hypothesis. In a series of 41 mRCC patients, tumour hypoxia was assessed before the start of sunitinib and after one month of sunitinib by an ^{18}F-fluoromisonidazole positron emission tomography (PET)-CT-scan. ^{18}F-fluoromisonidazole accumulates intracellularly in hypoxic cells. At baseline, tumour hypoxia as assessed by ^{18}F-fluoromisonidazole PET/CT uptake in mRCC was less frequent and less pronounced than initially suspected. Patients with initially hypoxic targets had shorter PFS than patients with non-hypoxic targets. OS was comparable between hypoxic and non-hypoxic patients, but the median follow-up was probably too short to notice differences. Under therapy with sunitinib, ^{18}F-fluoromisonidazole uptake significantly decreased in initially hypoxic target metastases but did not increase in targets which were initially normoxic: the latter metastases did not become hypoxic (152).

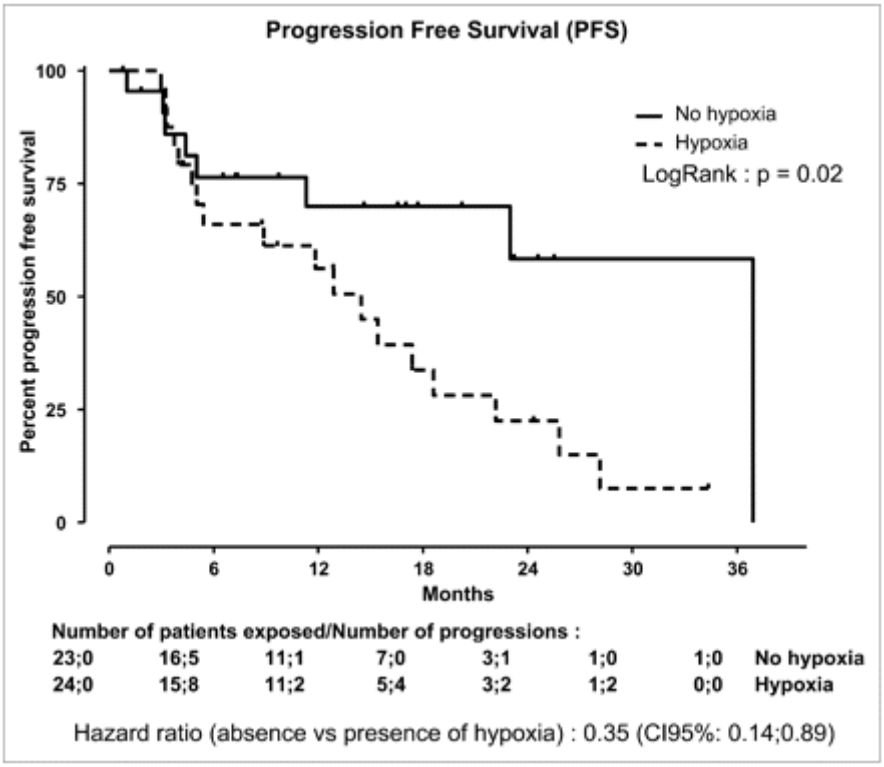

Figure 1.: 75% of patients with hypoxic metastases were free of progressive disease at 4.8 months (95%CI, 2.99–11.83), compared with 11.3 months (95%CI, 3.08–36.9) for other patients (p = 0.02). Courtesy of Hugonnet et al. (152)

- Among our four expression-profile based subgroups ccrcc1 to ccrcc4, there was an important difference in expression of pathways associated with cellular response to hypoxia. The good prognostic ccrcc2- and ccrcc3-subgroups had a lower expression of these pathways, meanwhile the poor prognostic ccrcc1- and ccrcc4-subgroups displayed an important expression of these pathways.

2.3. The balance normoxia *versus* hypoxia: the trigger of response to anti-VEGF-targeted therapy?

Therefore, our hypothesis is that the balance normoxia *versus* hypoxia or neo-angiogenesis *versus* hypoxia could be the ultimate trigger of response to anti-VEGF-targeted therapy.

- A tumour with important neo-angiogenesis, but with reduced hypoxia, will respond to anti-VEGFR-targeted therapy, because normoxia can be restored. On the opposite, a tumour that has an important hypoxia, in presence or absence of an important neo-angiogenesis, will stay hypoxic, even if anti-VEGF-targeted therapy could normalize the neo-vasculature.
- Hypoxia will be more important and more difficult to reverse in aggressively growing tumours and in important tumoral masses. This is consistent with the findings that tumour burden is associated with poor outcome in m-RCC patients treated with anti-VEGF-targeted therapy (128, 129): the larger the tumoral lesions, the higher the intratumoral hypoxia.
- Tumours with EMT, which is induced by c-myc and can be associated with a sarcomatoid phenotype on microscopy, switch to a hypoxia-resistant growth modus, with a reduced need for neo-angiogenesis. In these tumours, reduction and normalization of the vessels will not affect tumour growth.
- In ccRCCs, due to frequent VHL-impairment, there is an important neo-angiogenesis compared to the relatively low importance of the hypoxia. Therefore, in opposition to other tumour types, anti-VEGF-targeted therapy can work in ccRCCs in monotherapy.

Assuming that the reduction of neo-vessels leads to tumoral reduction with restoration of normoxia, the question remains whether the communication between endothelial cells and tumoral cells is merely oxygen pressure, or if there is any other tumour stimulating growth factor that would be secreted by endothelial cells.

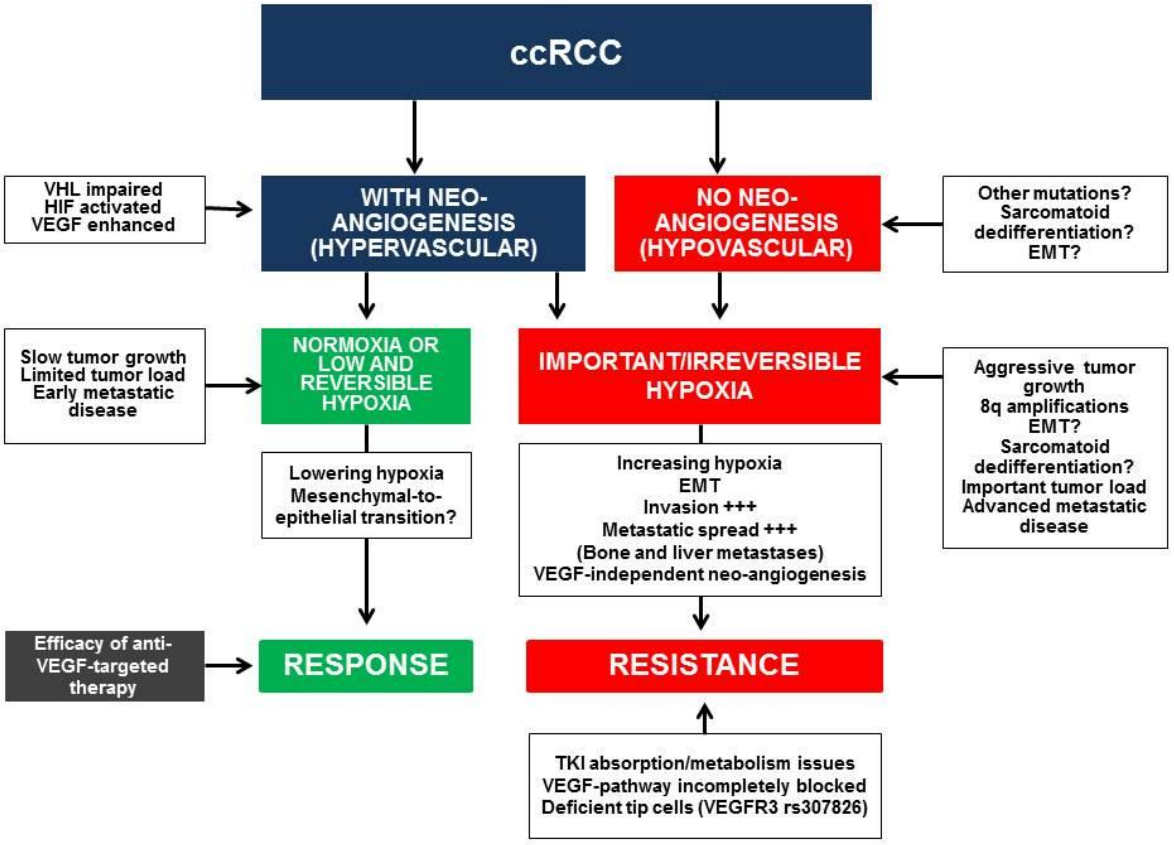

Figure 2: This scheme explains the hypothesis that sensitivity/resistance to anti-VEGF-targeted therapy in m-ccRCC might be the result of the balance neo-angiogenesis/hypoxia.

3. Future research perspectives

3.1. Expression-profile based ccRCC classification

The analyse of additional primary nephrectomy specimens in order to validate the impact of the expression-based ccRCC-classification as a prognostic and predictive marker is foreseen. This additional tumour series will allow us to enrich each subgroup of tumours with additional samples in order to increase the power to detect specific mutations, molecular characteristics and pathogenic events in each subgroup. Good candidate genes for additional mutation analysis, given the significant differences in epigenetic characteristics in each subgroup, are genes associated with chromatin modification such as *BAP1*, *SETD2* and *UTX*. Until today, no association between the mutation, hyper-methylation or LOH of *VHL* and outcome on anti-VEGF-targeted therapy has been shown, although the lack of functional VHL-protein could stabilise HIF and thus lead to VEGF-mediated neo-angiogenesis (140, 141). Therefore, we will study alternative VHL-impairment mechanisms such as *transcription elongation factor B (TCEB1)*-mutations (40) and alternative VHL-independent HIF-stabilisers such as the oxygen sensors prolylhydroxylase (PHD, also called egl-9 family hypoxia-inducible factor-1 or EGLN1) and factor inhibiting HIF (FIH) (153). We will take a closer look at EMT and at the interaction between endothelial cells and tumour cells with special attention for angiocrine factors that might influence tumour growth. Finally, we will study the impact of HIF1-alpha and HIF2-alpha on cell cycle induction and outcome.

As the efficacy on anti-VEGF-targeted therapy is in most cases based on the response of the metastases on CT-scans and as these metastases often appear several years after resection of the initial tumor, molecular analysis of metastatic sites is warranted. More precisely, we will analyse the stability of the expression-based ccRCC classification through the molecular characterisation of metastasectomy specimens.

Additionally, we intent to study if the expression-based ccRCC classification could help in the decision to perform a metastasectomy. In fact, after initial nephrectomy, or at the moment of initial diagnosis of the kidney tumour, patients can present with a single metastasis. The clinicians have then to decide if they perform a metastasectomy or not. This intervention being only useful if the patient will not rapidly recur with new and multiple metastasis. At the present moment, only clinical and biochemical criteria are available to help in the decision.

3.2. Germ-line polymorphisms

Given its possible predictive value, we intent to validate the impact of SNP rs9582036 in *VEGFR1* on outcome in m-ccRCCs treated with anti-VEGFR-TKIs. The study of polymorphisms in the *VHL*-gene, known to have a functional impact, will help us to understand better the impact of VHL on outcome in m-ccRCC patients treated with anti-angiogenic drugs. We plan to validate the other associations between polymorphims and outcome (SNPs in *VEGFR3, NR1/2, NR1/3, ABCB1*) in a prospective way and to combine them in a scoring system. The impact of polymorphisms in genes involved in sunitinib pharmacokinetics on sunitinib plasma levels will be

studied. Finally, we intent to study the association between the presence of *VEGFR1* and *VEGFR3* polymorphisms and the level of expression of these proteins in tumoral samples as well as micro-vessel density.

3.3. Response prediction in second-line therapy

As a consequence of the availability of several anti-VEGFR-TKIs and mTOR-inhibitors, several sequential combinations are possible. Two phase III studies showed a benefit of everolimus (compared to placebo) and axitinib (compared to sorafenib) in the second-line metastatic setting (6, 20). Nevertheless, response on second-line therapy is generally poor with a mPFS of 3-4 months for everolimus and sorafenib and 6 months for axitinib. Long lasting responses and important tumour shrinkage, as seen in the first-line setting, are seldom seen in second-line therapy. Moreover, an important part of the patients are progressive after only two months of therapy, which is comparable to the poor results observed in placebo-treated patients. The comparison of a switch to mTOR-inhibitors or the continuation of VEGFR-blockade in the second-line setting after progression on a first-line VEGFR-inhibition, is ongoing, but provisional data does not seem to show important differences. Therefore, we would like to study factors associated with outcome in the second-line setting in order to identify who could be the patients with a potential benefit of a second-line therapy. This study can have important clinical implications: it can identify patients who only will have side effects and no benefit. Moreover, given the high costs of targeted therapies, the identification of a group of patients resistant to second-line targeted therapies can also have a beneficial financial impact for the health insurance system.

3.4. Bone metastases and outcome

We will pursue our research on the impact of the presence of bone metastases in m-ccRCC.

Since January 2012, patients starting on anti-VEGFR-TKI therapy at the University Hospitals Leuven, undergo a baseline whole-body MRI (WBMRI) in order to study if this new imaging technique can improve the detection of bone metastasis, compared to standard investigations such as CT-scan and bone scintigraphy. In patients with bone metastases, by serial WBMRIs during therapy with anti-VEGFR-TKIs, we also want to document if targeted therapy is as helpful in bone metastasis as it is in other metastatic sites.

Finally, we want to validate in a prospective observational trial our data on benefits and risks of concomitant TKI and bisphosphonate use in m-ccRCCs with bone metastases. A randomized study of anti-VEGFR-TKIs with bisphosphonates *versus* anti-VEGFR-TKIs with placebo, although meaningful, is difficult to implement for ethical reasons because bisphosphonates are important in preventing skeletal related events, which are particularly frequent in mRCCs with bone metastases. Not only patients treated with bisphosphonates will be considered, but also patients treated with other bone sparing drugs like denosumab.

ABSTRACT OF THE RESEARCH

Based upon the knowledge that neo-angiogenesis plays a critical role in the progression of locally advanced and metastatic ccRCC, efficient drugs targeting the VEGF-pro-angiogenic pathway were developed during the last decade.

These drugs, mainly anti-VEGFR-TKIs, which are now increasingly used in clinical practice, allow to elicit responses and to prolong survival in mRCC. Unfortunately they can induce severe side-effects and are very expensive. Our project was to identify/discover clinical factors, pathological and biochemical characteristics, and new molecular markers allowing to predict response and outcome in m-ccRCC patients treated with anti-VEGFR-TKIs. Additionally, independently of the systemic treatment used, we also investigated whether some of these factors were of prognostic value in m-ccRCC patients.

We identified as clinically relevant for prognosis in m-ccRCC patients the following pathological and biochemical features: (A) The presence of bone metastases, with or without other metastatic localizations, generally indicate a poor prognosis. (B) Elevated baseline CRP-levels and (C) the presence of an important component of sarcomatoid dedifferentiation in the primary tumour and/or the metastasis are also associated with a more aggressive behaviour and worse outcome.

We also found several biochemical markers with a (potential) predictive value, among them variants in the ABCB1-efflux pump and in the VEGFR1 and -3.

These newly identified clinico-pathological and biological characteristics of m-ccRCC patients and of their tumors, as well as other markers described in the literature, allow us to predict more precisely the outcome in an individual patient treated with anti-VEGFR-TKIs. m-ccRCC may respond to targeted anti-angiogenic therapy, but m-ccRCC with an important sarcomatoid component are nearly always refractory. We showed that m-ccRCC patients with bone metastases benefit from agents blocking the osteoclastic activity like bisphosphonates. When the latter or newer agents like denosumab are combined to anti-VEGFR-TKIs, better results can probably be achieved, but caution and preventive measures are warranted in view of an increased risk of osteonecrosis of the jaw.

Moreover, based on a multi-omics analysis using unsupervised clustering of expression data, we identified four molecular subgroups of ccRCCs, each of them displaying distinct and typical histologic, mutational, epigenetic, and cytogenetic characteristics. When treated with the anti-VEGFR-TKI sunitinib, these four subgroups behaved differently in terms of RR, PFS and OS: two subgroups displayed a good outcome with high RRs, while the two remainders had low RRs and very short survival. As a consequence, this new molecular ccRCC classification distinguishing these subgroups has certainly a prognostic value; further prospective validation of its predictive interest is warranted and ongoing, in order to see whether anti-VEGF-targeted therapy could be selectively indicated or omitted in some of them.

Finally, our findings are hypothesis-generating, contributing to a better understanding of the mechanisms of sensitivity or resistance of m-ccRCC to anti-VEGFR-TKIs. These drugs block further development of neo-angiogenesis, and may even restore normal vasculature in m-ccRCC, leading to better oxygenation of tumoral deposits, thereby interrupting the vicious circle of hypoxia-induced tumor progression. This is the case in many m-ccRCC, while sarcomatoid tumors, which generally display little or no neo-angiogenesis, are tumors progressing

under irreversible hypoxic conditions. We thus emit the hypothesis that response in m-ccRCCs treated with anti-VEGFR-TKIs is highly dependent of the balance between neo-angiogenesis and hypoxia.

SAMENVATTING

Tot enkele jaren geleden was het gemetastaseerd heldercellig niercelcarcinoom een erg moeilijk te behandelen ziekte. Sinds 2005 beschikken we echter over een aantal nieuwe geneesmiddelen om deze ziekte te bestrijden. Deze geneesmiddelen trachten de aanmaak van bloedvaten in tumoren tegen te gaan en worden bloedvatremmers genoemd. Opdat een tumor of de uitzaaiingen van een tumor zouden kunnen groeien, zijn er immers nieuwe bloedvaten nodig die zuurstof en energie aan de kankercel kunnen bezorgen. In de jaren zeventig reeds ontstond het idee om tumorgroei te vertragen door nieuwe en bestaande bloedvaten te blokkeren.

Het heldercellig niercelcarcinoom is precies een ziekte waar er een belangrijke aangroei is van nieuwe bloedvaten. Mogelijks is het daarom dat bloedvatremmers de meest efficiënte therapie ooit zijn in de behandeling van deze ziekte. Sunitinib, pazopanib, axitinib, sorafenib en bevacizumab worden nu routinematig met succes gebruikt bij de behandeling van het gemetastaseerd heldercellig niercelcarcinoom. Ongeveer 40% van de patiënten die met bloedvatremmers behandeld worden, ondervindt een regressie van de tumoren. Bij een andere 40% kan de ziekte worden gestabiliseerd. Bij 20% van de patiënten blijken deze nieuwe therapieën echter niet werkzaam. Bovendien zullen de meeste patiënten, bij wie in eerste tijd een stabilisatie of regressie van de tumor wordt vastgesteld, uiteindelijk terug hervallen: hun tumor en uitzaaiingen beginnen opnieuw te groeien binnen een periode van gemiddeld ongeveer één jaar.

Alhoewel deze geneesmiddelen reeds een tiental jaren gebruikt worden, is hun werkingsmechanisme nog niet volledig ontrafeld en is er bijzonder weinig gekend over de mechanismen van resistentie. Dit onderzoeksproject had als doel deze vragen te beantwoorden. De resultaten van dit onderzoek moeten ons in de toekomst helpen om het juiste geneesmiddel te geven aan de juiste patiënt zodat we een maximale efficiëntie kunnen bereiken bij patiënten bij wie deze geneesmiddelen werkzaam zijn. Aan patiënten bij wie deze geneesmiddelen niet efficiënt zijn, zullen andere, nog te ontwikkelen geneesmiddelen moeten worden voorgeschreven. Zij zullen de bloedvatremmers niet meer voor niets innemen en bespaard blijven van bijwerkingen. Dit onderzoek zal op termijn dan ook bijdragen tot het beter benutten van de beschikbare financiële middelen in de gezondheidszorg.

In ons onderzoek hebben wij verschillende factoren gevonden die de werkzaamheid van deze geneesmiddelen gedeeltelijk kunnen verklaren.

- We konden aantonen dat de aanwezigheid van botmetastasen een ongunstige prognostische factor is. Aansluitend toonden we aan dat het toevoegen van botversterkers de efficiëntie van de therapie kan verbeteren.
- We toonden aan dat varianten in genen betrokken bij de absorptie in de darm en de metabolisatie van sunitinib een invloed kunnen hebben op de efficiëntie van de therapie. Deze varianten geven aanleiding tot een betere absorptie of tot minder afbraak van het geneesmiddel.
- We beschreven de impact van varianten in eiwitten betrokken bij de aanmaak van nieuwe bloedvaten, meer bepaald in de VEGF-receptoren- 1 en -3, op de resultaten bij behandeling met sunitinib.
- We hebben aangetoond dat slechtere resultaten bekomen worden onder behandeling met sunitinib bij patiënten met een inflammatoire toestand (een hoog C-reactief proteïne) bij aanvang van de therapie.
- De aanwezigheid van een component van sarcomatoïde dedifferentiatie in de primaire tumor van 25% of meer van het totale tumor-volume is ook een negatieve prognostische factor onder behandeling met bloedvatremmers.

- Tenslotte waren we in staat om het heldercellig niercelcarcinoma in te delen in vier subgroepen op basis van moleculaire kenmerken, en aan te tonen dat twee subgroepen geassocieerd zijn met een met goed resultaat en twee subgroepen met een slecht resultaat onder behandeling met sunitinib.

SHORT DESCRIPTION OF PROFESSIONAL CAREER AND PUBLISHED ARTICLES

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Born in Leuven (Belgium) on the 26th of July 1970.
Address : Groot Begijnhof 41-0201, 3000 Leuven
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Education

1982-1988 High School: Sint-Pieterscollege Leuven: Latin-Greek.
1988-1991 Bachelor in Medicine FUNDP Namur.
1991-1995 Master in Medicine UCL Brussels.

- Graduation in June 1995 “Summa cum laude”.
- January-June 1995 : 5 months of full-time fundamental research at the Ludwig Institute for Cancer Research (Prof. Boon), part of the Institute for Cellular Pathology (ICP). Research on genes coding for melanoma-specific antigens.

Professionnal experience

1995-2003: Project-manager in the NGO ACTEC

- ACTEC is a Non-Gouvernemental Foundation for Development Cooperation, approved by the Belgian Government and the European Commission to finance development projects in third world countries.
- Management of projects in health, social promotion, education and microfinance in Central and South America, Central and Western Africa and Lebanon.

2003-2009: Training in Internal Medicine and Medical Oncology Katholieke Universiteit Leuven and University Paris-5 René Descartes

- Virga Jesse Ziekenhuis Hasselt (October 2003 - September 2004) and Universitaire Ziekenhuizen Leuven (October 2004 - March 2009)
  - 21 months of training in Internal Medecine (Cardiology, Gastro-enterology, Pneumology, Intensive care)
  - 33 months of training in Medical Oncology: 4 months of Haematology, 3 months of Senologie (Prof. Robert Paridaens), 2 months of Thoracic Oncology (Prof. Johan Vansteenkiste), 4 months of Digestive Oncology (Prof. Eric Van Cutsem), 3 months at the Phase I clinical trial unit (Prof. Patrick Schöffski), 3 months of Urogenital Oncology and Sarcoma, 6 months at the Outday Clinic, 5 months Head-and-Neck, Neuro-Oncology and Melanoma, 2 months at the Palliative Care Unit, 1 month Gynecological Oncology (Prof. Ignace Vergote)

- Hôpital Européen Georges Pompidou Paris (April 2009 – September 2009)
  - Outday Clinic and specialised consultation in Urogenital Oncology (Prof. Stéphane Oudard)
  - Clinical research on anti-angiogenics (Prof. Stéphane Oudard).
  - Translational research on genetical predictive factors for response on anti-angiogenics in clear cell renal cell carcinoma (Prof. Jessica Zucman-Rossi INSERM U674).

— 30th September 2009 : Master in Medical Oncology

— Additional training: ESO Masterclass in Clinical Oncology, Ermatingen, Switzerland, March 2010. EORTC course on statistics, Brussels, Belgium, June 2011.

October 2009 – July 2010: Staff member Departement of Medical Oncology Hôpital Européen Georges Pompidou and research unit INSERM 674 « Génomique fonctionnelle des tumeurs solides », University Paris-5 René Descartes (Paris - France)

— Clinical practice in Urologic Oncology
— Preclinical and clinical investigation in Urologic oncology

August 2010 - …: Staff member Department of General Medical Oncology University Hospitals Leuven, KULeuven, Leuven – Belgium and invited researcher at INSERM U674 « Génomique fonctionnelle des tumeurs solides », University Paris-5 René Descartes (Paris - France)

— FWO Vlaanderen Pre-Doctoral Grant 2011-2013

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